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Possibilities of Improved Therapy for Cancer Patients[1]

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It is indeed an honor to have been invited by Dr. Cowdry and the Board of the Barnard Free Skin and Cancer Hospital to give the second Barnard Hospital Lecture, particularly because St. Louis is one of the oldest and most important centers of experimental and clinical cancer research in the country.

Since I am a laboratory worker, it was with some misgiving that I agreed to speak to you on the possibilities of improved therapy for cancer patients. However, I shall deal mainly with fundamental principles of therapy, and these are essentially the same, whether they arise in the clinic or in the experimental laboratory. It is my aim to discuss, briefly and as objectively as possible, various points which to my mind may lead to improvements in cancer therapy. These may be grouped into two broad classes: first, the more widespread application of known methods and their improvements; and, second, the development of new methods.

[1] *Barnard Hospital Lecture read before the St. Louis Medical Society, November 19, 1940. Reprinted with permission from the Editor of the Journal of the American Medical Association.*

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PROFESSIONAL AND LAY EDUCATION

Cancer, both from the etiologic and the therapeutic standpoint, is a unique disease, or rather, as I never neglect to stress, a group of distinct diseases with a few common characteristics. One may almost go so far as to state that the malignant transformation of each type of body cell endows the resulting neoplasm with its own peculiar biological character. This and other facts account for the great diversity and intricacies of present techniques of treatment. It is not surprising, therefore, that cancer therapy has become more and more a specialty, the mastery of which requires years of experience.

There is also the curious paradox that in spite of the high total cancer mortality, i. e., 150,000 deaths in 1938 for the country as a whole, the average practicing physician sees only very few cancer patients each year. Hence, the need for an adequate number of physicians and surgeons experienced in the diagnosis and treatment of the various types of cancer is quite obvious. In order to assist in supplying this need the National Cancer Institute during the last 2 years has given 44 care-

fully selected physicians and surgeons "traineeships" in cancer clinics at Government expense for a period of 1 or more years. It is to such training centers as your Barnard Hospital that these trainees are sent with the understanding that they will practice what they have learned on completing their period of study. But, as you readily see, this relatively small number of trained men will not supply the urgent need. It is, therefore, encouraging that many medical schools are becoming more "cancer conscious" and are improving the training of students in the principles of cancer diagnosis and treatment.

Another point I wish to make is the need for readily accessible diagnostic and treatment centers throughout the country. The American College of Surgeons has approved 345 hospitals which meet the prescribed standards. Eight States have no approved clinics. This means, of course, that cancer patients in such areas either do not get proper attention or have to travel long distances at considerable expense.

Improvement in the treatment of cancer should begin with a more widespread education of the public concerning the early symptoms of the disease. This is being carried out by official and private agencies. In a recent paper (*1*), McDowell, of the United States Public Health Service, gives the results of a survey in the Pittsburgh area on the incidence of cancer on the basis of case records and death certificates for the year 1937, including 6,103 cases Fifty-nine percent of fatal cases had been diagnosed as malignant less than 6 months before death, and 80 percent of them less than a year. Since it is almost axiomatic that the probability of cure of most types of cancer increases with early diagnosis, it is obvious from this illustration that much remains to be done in the way of lay education, especially in view of the fact that 6 approved cancer clinics are located in the Pittsburgh area.

DIAGNOSTIC METHODS

It is also quite obvious that there is great need for improvement of diagnostic methods for internal cancer. The introduction and development of the roentgenological method during this century has revolutionized the diagnosis of cancer, but it must be admitted that many kinds of *early* neoplasms escape detection by this method. In this connection it is of interest to call attention to the possibilities of the gastroscope as a new tool for the diagnosis of gastric cancer. This was clearly brought out during the recent conference on gastric cancer. Brief mention should also be made of the various serological tests which have been proposed in the past. At this time it seems improbable that complement-fixation and precipitation tests will be of practical value. For one reason it is very difficult to prepare sufficiently specific antigens from different kinds of cancerous tissues. Freund and Kaminer developed a cytological test on the principle that normal serum will cytolyse cancer cells while cancer serum will not. Other workers have failed to demonstrate the usefulness of this test. The tests devised by Fuchs and Abderhalden are based on the assumption that proteolytic enzymes of cancerous tissues are released into the blood and can be demonstrated by chemical methods. A limited experience with these tests indicates that they are fairly reliable in advanced cases, where other methods are better, but fail in the diagnosis of early cancer. However, it is within the realm of reason that future systematic work on the enzymes of cancerous tissues may furnish the basis for diagnostic tests of certain specific neoplasms. The occurrence of prolan in the

urine with enlargement of the testicle without discernible cause is of considerable value in the diagnosis of teratoma, although the earlier reports have not been fully confirmed by more recent work. Encouraging progress is being made in the use of serum phosphatase in the diagnosis of bone tumors.

Much would be gained by more reliable information concerning so-called precancerous lesions. As an illustration, I again refer to the gastric cancer conference. It was brought out very clearly that much further work must be done to conclusively prove that chronic atrophic gastritis, polyposis, and possibly peptic ulcer predispose to gastric cancer. Clarification of this problem would undoubtedly result in earlier diagnosis and therefore a better chance for cure of this highly fatal disease. Progress in this field will come not only from clinical studies but also, I believe, by work on properly chosen animals as closely related to man as possible, as for instance dogs.

ROENTGEN AND RADIUM THERAPY

We may now pass over to a consideration of improvements in present therapeutic methods. It is not necessary to point out the tremendous development of surgical and radiological methods, each having its usefulness in the treatment of different types of neoplasms. Concerning radiation therapy Coutard, in 1937, wrote as follows: "Rich only in hope, possessing only incomplete information, incapable of offering precise techniques adapted to diverse types of cancer, radiotherapy has, however, obtained definite cures in cases incurable by surgery." This statement comes from a leader in this field and represents probably an understatement of the value of radiotherapy; it is evident, nevertheless, that there is an urgent need for more precise knowledge on the biological action of X-rays and radium on tumors, and normal cells, and normal tissues.

You are aware that the physics of ordinary X-rays and gamma rays of radium is well understood. These electromagnetic radiations represent energy of short wavelength traveling at fast speed comparable with that of visible light. When these radiations act on the atoms of gases they produce different effects, according to the wavelength used. A simplified presentation of an atom shows that it is composed of a nucleus and the surrounding electrons arranged in orbits. In one case, an electron may be ejected from an inner shell of the atom, and in addition the electron may be given a varying degree of kinetic energy. In another case, absorption of the energy may not only remove an electron from an orbit, but a portion of the energy may be transformed into radiation of lower energy. This is known as the Compton effect.

Now let us consider for a moment what may happen when these high-energy radiations instead of acting on simple gases are allowed to act on biological material, whether free-living cells or highly organized tissues. First, it is well to bear in mind that any mammalian cell represents an enormously complex physico-chemical system made up of complex high molecular material as proteins, lipids, carbohydrates, enzymes, and many other substances, all of which are arranged in a definite spatial pattern suitable for biological function. It is at once evident that the chemical changes induced in cells by X-rays and gamma rays may be infinite in numbers, yet it is reasonable to assume that they are not of equal biological significance. In other words, it is possible that the cell response to radiation may be more or less *selective* in a biochemical sense. The best support for this assumption comes from the artificial production of mutations by means

of X-rays. And some geneticists believe that the nucleoproteins of chromosomes may be specifically involved in the mutation process. This does not mean that the radiation effect is confined to nucleoproteins, as it is quite conceivable that highly important cellular enzymes may be destroyed. At all events this important field has not been sufficiently explored to furnish an adequate answer for the biochemical mechanism responsible for the biological action of these radiations. On the other hand, there is a considerable body of evidence furnished by clinical observation and laboratory experimentation concerning the histological, cytological, and functional changes induced in living material. Since you are familiar with the clinical results I wish to refer to some experimental work by Dr. Henshaw (*2*, *3*) of the National Cancer Institute. He exposed unfertilized sea urchin eggs to measured doses of X-rays and found that on subsequent insemination the first cell cleavage was much delayed as compared with that of nonirradiated eggs. Increased dosage increased the delay in first cleavage. If the dosage was kept constant, but the time between irradiation of the unfertilized egg and insemination was increased, the delay in cleavage decreased. This clearly indicates that within limits the egg *recovers* from the effects of irradiation. Further studies by Henshaw showed that irradiation of sperm before fertilization induced not only delay in cell division but also multipolar cleavage and disturbances in phase relationship of mitosis in the blastomeres.

When we consider the past work done on radiotherapy of malignant animal tumors it is at once apparent that the situtation is far more complex than in the case of free-living cells. The studies of Cramer (*4*), Mottram (*5*), Sugiura (*6*), and others indicate that the regression of a malignant tumor is initiated in part by a damage to the malignant cells which in turn elicits a process of repair beginning with an invasion of the tumor by macrophages. The transformation of these macrophages into fibroblasts then gradually leads to the formation of connective tissue replacing the malignant cells. Crabtree and Cramer found that in the absence of oxygen malignant cells appear to become more radio-resistant. This is of importance in case cancer cells as a result of treatment are enclosed in a relatively avascular fibrous tissue, thus interfering with their complete eradication by radiotherapy. Cramer's results, furthermore, suggest that the period of recovery of the carcinoma cells from *small* doses is surprisingly long, and apparently longer than the recovery period of many of the stroma cells. In 1930 Regaud and Ferroux (*7*) reported observations indicating a rapid recovery of the blood vessels of the skin from *small* doses. A year later Eidinow and Mottram (*8*) confirmed these results in work on the skin of rats, and Juul obtained similar results on mice. All these and other findings led to the now well-known fractional-dose technique. The principle of this method consists in the repeated application of relatively small doses in order to prevent any cumulative action on the normal tissues without losing the cumulating effect on the tumor cells.

This method receives further support from the comprehensive animal experiments of Murphy (*9*) on the function of lymphoid tissue. Large doses of X-rays cause an extraordinary depletion of lymphocytes followed by a more or less slow recovery. The depressed function of the lymphoid tissue seems to be correlated with a decreased resistance of the animal to the growth of tumors.

There is general agreement indicating that animal tumors irradiated *in vitro*

require almost twice the dose of X-rays to kill the cancer cells (shown by failure of producing tumors on transplantation into animals), as compared with the dose which is sufficient to cause regression of the same tumors within the animal.

Clinical experience has shown that the dosage has to be increased with increasing differentiation of the treated neoplasm. Another curious and as yet unexplained observation is the definite periodicity of the maximal radiation effect. This differs for different tissues. For the skin it reappears every 26 to 28 days, the same as the menstrual cycle; for the mucosa, it is half that time (Coutard (*10*)).

To sum up, it can be stated that present X-ray and radium therapy is the result of a vast amount of more or less empirical clinical experimentation supplemented by data secured by animal experimentation. It is quite evident, however, that we are very far from an adequate comprehension of the radiotherapeutic process. There is still no explanation for the apparent vast differences in radiosensitivity of the various normal and cancer cells. No concrete proof is available for the assumption that a given type of cancer cell is more radiosensitive than the normal cell of origin. Much further information is needed on the nature of the important recovery process from the effects of sublethal dosage. There can be little doubt that a better understanding of these complex problems would furnish a better rationale for cancer radiotherapy.

A subject which deserves mention concerns attempts to enhance the X-ray efficiency by adjunct therapy. Very little has been accomplished along this line. Preliminary observations on experimental tumors suggest that the intratumoral injection of sterile water increases the effectiveness of X-rays. Similar claims have been made for the combination of heat and X-rays. I believe that perhaps the most daring and yet promising attempt would be to alter the tissue metabolism by changes in diet, or by the administration of chemicals, in order to increase the radiosensitivity of the cancerous, relative to that of the normal tissues. Experimental work of this kind with tumor-bearing animals is now under consideration at our Institute.

Supervoltage X-Ray Therapy

We may now consider the possibilities of supervoltage X-ray therapy. The well-established fact that increase in the voltage of X-rays increases their penetrating power for tissues has led in recent years to the clinical use of X-rays of voltages from 400,000 to 1 million and slightly over. Hope was entertained that these powerful rays might greatly improve cancer therapy. It appears now, however, that the results of supervoltage therapy have been more or less disappointing. The recent careful studies of Packard (*11*) have shown that relatively little is gained, as far as the ratio of depth to surface dose is concerned, by increasing the voltage from about 400 to 900 kv. This is in agreement with the results obtained by Stone. In fact, Stone found the biological effect the same, if the same dose is delivered to the patient from opposite sides, the one side receiving 200-kv. X-rays and the other 1200-kv. rays. A few other investigators have made somewhat more favorable reports. Nevertheless, it appears at present that, while supervoltage X-rays may perhaps prove of value in the treatment of some deep-seated tumors, no great advance can reasonably be expected from this therapy.

Radio-Wave Therapy

For the sake of completeness brief mention may now be made of the possibilities of using radio-wave therapy. More than

10 years ago it was found that radiation of long wavelength, i. e., a few meters, such as used in long-distance broadcasting, produces severe physiological reactions. We (*12*) were among the first to show that the physiological effect was due to the production of heat within the irradiated living material. If experimental conditions were so arranged as to prevent an increase in temperature, no biological action was recognizable. Energy of this wavelength, acting upon normal or tumor tissue, increases the kinetic molecular energy and reveals itself as heat. The reason why this method has not proved useful is because malignant cells do not differ sufficiently from normal cells in regard to the critical temperature which causes their death.

Neutron Therapy

From time to time developments in the fundamental sciences have their profound repercussions on the cancer problem. The discovery of radium, for example, made possible the amazing revolution in nuclear physics which began some 20 years ago. In this short time the age-old alchemist's dream of the transmutation of the chemical elements has been materialized, and the so-called indivisible atom has been smashed into atomic particles, some of which are now being studied with regard to their possible value in the treatment of cancer.

It seems pertinent to introduce this subject by a consideration of the structure of the atom. The hydrogen atom is composed of the centrally located nucleus called proton, which has a positive electric charge and represents by far the largest mass of the hydrogen atom. In an outer orbit is located the negatively charged electron. The more recently discovered heavy hydrogen atom, called deuterium, is twice as heavy as ordinary hydrogen, because it has in its nucleus in addition to one proton, one neutron, a particle which is electrically neutral. The structure of the helium atom shows that its nucleus is composed of 2 protons and 2 neutrons, giving the atomic weight 4, and 2 negative electrons. It is evident that the heavier an atom is, the more complex is its structure. The heavy uranium, for instance, has 92 protons and 146 neutrons in its nucleus, besides 92 outer negative electrons.

The most powerful tool for the disintegration of atoms is the magnetic resonance accelerator (fig. 1), or cyclotron, developed by Lawrence and his coworkers. The cyclotron produces atomic particles of tremendous speed, which, when directed on chemical targets, cause the production of high-speed neutron rays or of artificial radioactive elements. The machine permits many successive accelerations of particles. For example, by giving a particle a hundred accelerations of 50 kv. each, particles of 5 million volts are obtained. The cyclotron consists of a powerful electromagnet, between the poles of which is placed a vacuum chamber containing two flat, hollow electrodes (fig. 2) connected with a radiofrequency oscillator. If the vacuum chamber contains hydrogen at low pressure, the proton *P* is whirled around gaining in speed each time it passes from one electrode to the other until it is finally discharged through the window *W*, as a beam of high-speed protons. Similarly, a beam consisting of high-speed deuterons is obtained if the vacuum chamber contains heavy hydrogen, called deuterium. Deuterons are nuclei consisting of one proton and one neutron. Through the courtesy of Dr. Cooksey, I am able to show a deuteron beam emerging from the California cyclotron, which was obtained by colored photography. When such a deuteron beam strikes a target of beryllium, gamma rays and neutrons are produced. By filtering out the gamma rays by lead, a nearly pure high speed neutron beam is

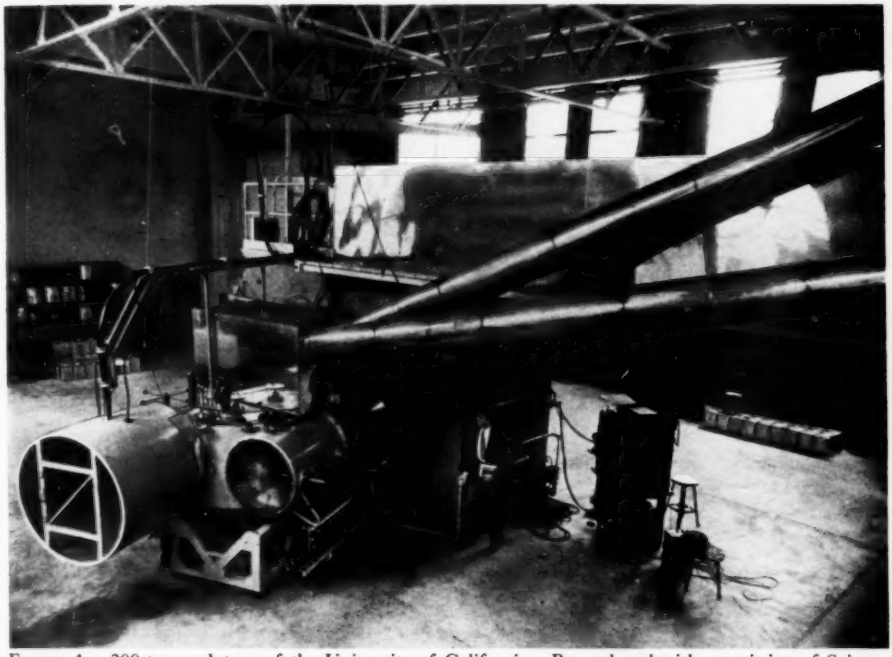

FIGURE 1.—200-ton cyclotron of the University of California. Reproduced with permission of Science Service.

obtained. Neutrons produce ionization, as do gamma rays and X-rays. I show you here photographs of Wilson cloud chambers, which demonstrate that neutrons produce much more concentrated ionization tracks than X-rays or gamma rays. Whether the characteristic ionization by neutrons seen in gases will also elicit specific biological effects on tumors and normal tissues remains to be determined. It has been established that neutrons react very strongly with organic material rich in hydrogen, in contradistinction to short X-rays. However, it happens that most organic constituents of tissues are relatively rich in hydrogen and, therefore, a selective chemical effect from neutrons is not so easily to be expected. At all events, the work of the Radiation Laboratory of the University of California, which is being supported by grants-in-aid from the National Cancer Institute, has shown that fast neutrons have a high penetrating power in human and animal tissues, quite adequate for deep therapy. The California studies and the similar projected studies of the Mallinckrodt Radiological Institute, which also have received financial support from the Public Health Service, will in time assess the value of this novel cancer therapy.

A useful unit for measuring neutrons is called the n unit. It is the exposure of neutrons that will cause in the Victoreen X-ray r meter the same reading as an exposure of 1 r of X-rays. Preliminary work suggests that the biological effect of 1 n on the skin is equivalent to that of 10 r of X-rays.

A few remarks may now be made on the interesting suggestion by Kruger (*13*) of using slow neutrons for cancer therapy.

Such neutrons pass through body tissues without the production of energetic recoil protons, and little or no tissue damage is done. If, however, the tissue is injected with boron or lithium the slow neutrons are captured by these elements with the release of very energetic atomic particles. These cause the localized destruction of the injected tissue. Zahl, Cooper, and Dunning (*14*) have just reported experiments showing that the injection of simple forms of boron or lithium into the transplanted mouse sarcoma 180, followed by irradiation of the whole mouse with slow neutrons, causes a significant increase in tumor regression. It is doubtful that in its present form this therapy will prove of considerable value. However, should it be possible to discover neutron-capturing and relatively nontoxic compounds which, administered, would be selectively absorbed by certain malignant tumors, then slow neutron therapy might well yield results of practical value. The discovery of suitable chemicals is unquestionably a major undertaking for which there is, as yet, no theoretical basis.

ARTIFICIAL RADIOACTIVE CHEMICALS

Another new approach to cancer therapy opened up by the development of the cyclotron is the possibility of the usefulness of artificially produced radioactive chemicals. The California radiation laboratory has taken the lead in this new line of investigation. Repeated intravenous injections of radiophosphorus into patients with leukemia have brought about long-lasting remissions in the disease, but it is too early to state whether this therapy is better than the customary treatment with X-rays. The theoretical foundation on which this new therapy is based is the fact that artificial radioactive elements are unstable and emit electrons or beta rays just as do radium and other natural radioactive chemicals. The radioactivity of the artificial radioactive chemicals is measured by the "half life," that is to say, the time required for half of the radioactivity to be emitted. The following tabulation shows the half life of a number of chemical elements. The last of these is radioiodine which has a half

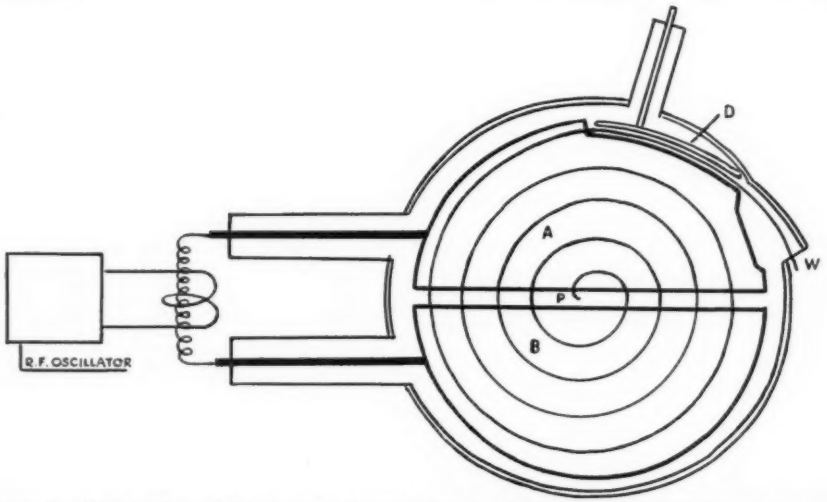

FIGURE 2.—Cross section of the vacuum chamber of cyclotron, containing two D-shaped electrodes, A and B.

life of 8 days and which emits gamma rays.

Chemical elements:	*Half life*
C^{11}	20.3 min.
N^{13}	10.5 min.
Na^{24}	14.8 hours.
S^{35}	80 days.
P^{32}	14.5 days.
Fe^{59}	47 days.
Ca^{41}	250 days.
I^{131}	8 days.

This can be used for illustrating another principle for a new approach to therapy. It is well known that thyroid tissue stores by far the greatest portion of the iodine of the body, and recent work by Hamilton and Soley has demonstrated the same for radioactive iodine (I^{131}). The question was raised, therefore, whether it is not possible to treat primary and metastatic malignant tumors of the thyroid by the administration of radioiodine. Unfortunately, the above-mentioned workers found that the cancerous thyroid tissues so far tested lacked the ability to accumulate radioiodine. Nevertheless, further endeavors based on this principle may well be repaid, not in anticipation of a cure-all for cancer, but perhaps for the discovery of a treatment of certain specific neoplasms.

PROTECTION AGAINST HIGH-ENERGY RADIATIONS

Before leaving the consideration of X-rays, gamma, and neutron rays, it is timely to call attention to a very practical topic, namely, the need for protection of clinical and laboratory personnel against these powerful physical agents. In the limited time it is not possible to discuss this subject as fully as its importance merits. First, let me say that it is perfectly feasible with relatively little financial expense to have the physical equipment and the operating methods so arranged as to reduce exposure to radium and X-rays to or below the present accepted maximum safe daily dose of 0.1 of 1 r.

The National Cancer Institute has loaned radium to some 48 clinics for cancer therapy. We naturally have a responsibility to insure that this radium does not cause health hazards to the personnel using it. A recent survey of such clinics by Dr. Scheele and Mr. Cowie has clearly revealed good conditions in many places, whereas, in an appreciable percentage of clinics, some of the personnel are exposed to an unsuspected high-radiation intensity. This not only applies to exposure from radium, but also to exposure of personnel to X-rays incident to diagnostic and therapeutic work. Without arousing unnecessary alarm, it is unquestionable that many physicians and dentists are not quite aware of the insidious nature of biological high-energy-radiation effects, and they would be surprised if they actually measured with a Victoreen chamber the radiation they receive for long periods of time.

It is established that the biological effectiveness increases with increasing dosage. The possible production of cancer, leukemia, and sterility is generally recognized, so is also the danger of producing an abnormal child following irradiation of the embryo *in utero*. But what is regarded by many physicians as highly improbable is the possible hazards arising from irradiation of the ovary or testis with the consequent production of genetic changes in the first and subsequent generations. We can readily appreciate the difficulties of demonstrating such genetic effects in clinical material. On the other hand, this should not permit us arbitrarily to dispose of the very large body of evidence secured by geneticists on plants and animals. Demerec recently stated:

> In a very few branches of biological science, the fundamental principles are as applicable to all living organisms as they are in genetics. It is a

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well-established fact that fundamental (genetic) principles discovered on plants hold for animals and vice versa. Man is no exception to that rule.

Manv of the mutations, produced by either chromosome breaks or by elimination of certain genes, are lethal and therefore self-eliminating. The danger lies in the production of mutations injurious to subsequent generations and expressed in morphological (visible) or functional (invisible) effects. I show here two photographs made by Dr. Henshaw, illustrating the results obtained by exposing frog sperm to 200-kv. X-rays followed by fertilization of normal eggs by the irradiated sperm. It is evident that the early proliferation of the egg is quite normal despite very large doses of radiation. However, it is apparent that relatively very small doses cause extensive abnormalities in differentiation, which increases in frequency and degree with increasing dose. The first effect is apparently a probability reaction, whereas the second appears to be a progressive change in each individual embryo.

Experimental evidence also indicates that genetic changes induced by radiation are proportional to the dosage used, and this undoubtedly differs in different species. In view of the importance of this whole question, the National Cancer Institute is conducting a comprehensive investigation with the advice of such leading geneticists as Muller and Demerec. In all events it is not only advisable but perfectly practical with little trouble (1) to prevent the exposure of the genitals of operators to direct or stray radiation, and (2) to avoid similar exposure of patients during therapeutic or diagnostic procedures by proper shielding of the primary sex organs.

Neutron therapy is so new that very little is known about its hazards. Protection is afforded by surrounding the cyclotron by large tanks of water which absorb neutrons, and a control room far removed from the cyclotron. Skin lesions have been noted similar to those produced by sunburn. Tuve, of the Carnegie Institution, insists in keeping exposure in his laboratory down to the equivalent of 0.01 of 1 r per day. Great caution is certainly indicated in the operation of these tremendously powerful machines.

SYSTEMIC CANCER THERAPY

The great problem yet to be solved, as I have already intimated, is the discovery of effective systemic treatments. Surgery, X-rays, radium, and in all probability neutrons are measures which can cure cancer only by the complete destruction of more or less localized malignant tumors. They fail completely to bring about cure in the more advanced disseminated stage, because radiation therapy uses a technique which is not based on a truly selective biological radiation effect. In view of the vast amount of money and energy invested in the development of present-day radiation therapy, it would seem that a corresponding investment for the search of selective therapy is fully justifiable. There are essentially two approaches to this problem, namely, the rational and the empirical method, or a combination of the two. Rational therapy of any disease, as, for example, the serum treatment of infectious diseases, depends on the comprehension of the cause and nature of the disease in question. As concerns human cancer, the best available evidence indicates that it is not due to a specific infectious agent. This practically eliminates the probability of finding a specific vaccine or serum. Both clinical experience and extensive study of cancer in animals strongly suggest that cancer is caused by the transformation of the normal cells of the body into cancer cells through the operation of a great diversity of factors and

agents operating from within or from without the organism. As far as we know now the malignant transformation of cells is irreversible and expresses itself in a varying degree of dedifferentiation and increased rate of cellular proliferation. What we need to know, in order to discover a rational specific therapy, is (1) why the biological behavior of cancer cells differs from that of the original normal cells, and (2) what kind of biochemical differences account for the differences in biological behavior. Such fundamental questions can only be answered on the basis of a much better understanding of cell physiology and biochemistry, as well as the functional and chemical interrelation of the various tissues and body fluids. We have reason to hope that this approach will be fruitful given sufficient time.

The empirical or trial-and-error method has already given us radiation therapy for cancer. A large part of the most valuable chemical therapeutic agents have been discovered purely empirically, beginning with cinchona for malaria to sulfanilamide and related drugs for the astonishing treatment of certain infections. Then there are examples where a partial understanding of the nature of a disease combined with physiological knowledge and empirical trial has led to success, as, for example, the discovery of the use of liver in the treatment of pernicious anemia.

Let me emphasize that it is hazardous for anyone to predict that a given scientific problem is insoluble for all time, as long as there are ideas which can be tested by sound experimentation. This is the lesson to be learned from the history of science. But, of course, no one can predict results or when they may come.

I believe that therapeutic studies on cancer should in the beginning be carried out on tumor-bearing animals where conditions can be accurately controlled. There is available a great variety of spontaneous, chemically induced, and transplanted tumors, and the list is constantly increasing. The remaining time will be given over to a brief summary of certain studies in experimental therapeutics.

The remarkable capacity of malignant tumors for apparently unrestricted proliferation suggested to us attempts to retard or possibly to completely inhibit cancerous growth by maintaining tumor-bearing animals on diets deficient in components essential for cellular proliferation. Classical nutrition experiments have shown that normal growth, and therefore tissue proliferation, does not occur unless the diet contains an adequate amount of all the so-called indispensable amino acids, the building stones of body proteins. It, therefore, was of interest to ascertain whether the growth of malignant tumors could also be inhibited by diets deficient in certain indispensable amino acids. Contrary to expectations we (*15*) were able to accomplish this in work on a typical spontaneous mammary carcinoma of the mouse. Table 1 shows the composition of a diet deficient in the amino acid, lysine; and the same diet supplemented by an adequate quantity of this amino acid. Young normal mice, when placed on the deficient diet, fail to grow without manifest signs of ill health. Soon after lysine is added to the diet, the mice begin to grow at a rapid rate. The growth rate of the mammary carcinoma is strikingly retarded by maintaining animals for several weeks on the lysine-deficient diet. Proof that this retardation of cancerous growth is due to a specific lysine deficiency is shown by the rapid tumor growth following the feeding of lysine. Similar results were obtained with a diet consisting of 30 percent whole milk powder and 70 percent ground wheat, which was made deficient in lysine by a preliminary heat treatment of the milk powder. Less

striking inhibition of tumor growth was obtained with a diet deficient in tryptophane.

TABLE 1.—*Composition of diet deficient in lysine, and with lysine added*

Gliadin diet		Gliadin diet plus lysine	
	Percent		*Percent*
Gliadin	18.00	Gliadin	16.80
Salt mixture	4.00	Salt mixture	4.00
B_1B_2	0.10	B_1B_2	0.10
Starch	54.00	Starch	54.32
Crisco	20.00	Crisco	20.00
Cod-liver oil	8.00	Cod-liver oil	3.00
		Lysine—2 HCl	1.20

Of particular interest are the experiments with diets deficient in sulfur-containing amino acids (*16*). For instance, in mice on a diet containing about 16 percent of whole milk powder as the principal source of protein, the tumors made very little growth. However, the tumors promptly began to grow rapidly on the addition of a small amount of cystine to the diet, or following the repeated subcutaneous injection of the cystine-containing peptide, glutathione (fig. 3).

To my mind these results, though not of immediate practical value, are of fundamental interest. Our own work and that of other investigators have furnished evidence of the great importance of the organic-sulfur system of the body: (1) In the regulation of enzymes controlling the degradation and synthesis of tissue protein; (2) in the control of cell proliferation; and (3) in the action of certain cancer-producing chemicals. This field should be cultivated in a far more comprehensive manner, due consideration being given not only to the proteins, but also to the numerous known biocatalysts of the tissues. It is at least permissible to speculate that the chemistry of cancerous tissues differs sufficiently from that of normal tissues as to influence selectively the growth of tumors by a deficiency or excess in certain biocatalysts. We may regard the human body as an organism which is marvelously coordinated not only anatomically and physiologically but also chemically. The whole trend of recent investigation is toward the concept of what might be called the "fluidity" of chemical organization, with a continuing chemical interchange between the anatomical units. There is no better illustration than the recent revolutionary changes in our conception of the protein metabolism of tissues and body fluids.

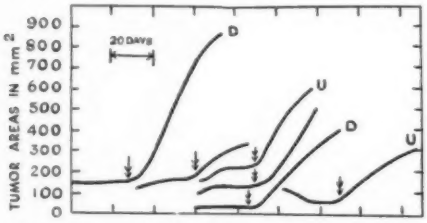

FIGURE 3.—Inhibition of growth of mammary carcinoma in mice maintained on a cystine-deficient diet, and stimulation of tumor growth following daily subcutaneous injections of glutathione (indicated by arrows).

Significant results have been secured by Murphy and Sturm (*17*). They found that repeated intraperitoneal injections of extracts of such normal tissues as embryo skin, placenta, or colostrum arrested the growth of mammary carcinoma in mice in about 70 percent of the treated animals, and in 22 percent the tumors regressed. These same extracts were ineffective when tested on mouse sarcoma 180. Such studies should be continued with a view to concentrating and isolating the active principles.

Another line of investigation in progress at the National Cancer Institute is based on clinical observations dating as far back as 1882. It has been noted by clinicians that certain intercurrent infections, particularly erysipelas, occasionally exercised

an apparently curative effect on certain malignant tumors. This led to the clinical use of bacterial filtrates, including Coley's fluid, which is a mixture of streptococcus and *Bacillus prodigiosus* filtrate. Favorable and unfavorable clinical results have been reported with various bacterial filtrates. This may be partially explained by the results obtained in animals, which indicate that the filtrate is effective on sarcoma, but apparently ineffective on other tumor types (*18*). Active filtrate fractions are obtained from *B. prodigiosus* grown on a synthetic medium, and present evidence suggests that the active principle may be associated with a bacterial polysaccharide (*19*). In large doses the preparations are toxic and kill the animals. Smaller doses produce striking macroscopic and microscopic hemorrhages in the tumors, followed in many cases by regression of the tumors. The hemorrhagic reaction appears to be specific for the tumor capillaries and is not evident in other tissue. Since the hemorrhage is accompanied by a marked reduction in the ascorbic acid content of the tumor, and since the injection of this vitamin prevents the hemorrhage (*20*), it is believed that the bacterial filtrate, by suddenly lowering the ascorbic acid of the tumor, so weakens the fragile new capillaries as to cause hemorrhagic extravasation. If the whole tumor tissue is affected, the tumor regresses. In patients filtrate elicits marked febrile reactions, and there are suggestions that the organism develops a partial tolerance, as indicated by the toleration of increasing doses. It is, of course, too soon to decide whether this form of therapy will be of practical value. The possibility is certainly not ruled out that improvements in the fractionation of the crude filtrate may furnish a preparation from which toxic, but therapeutically inactive, material has been eliminated, and which may have a sufficiently wide margin of safety between the toxic and hemorrhage-producing doses.

Another illustration of selective action of a chemical on tumor tissue is the observation made by Strong (*21*). Using spontaneous mammary cancer in mice, he found that the repeated oral administration of a synthetic chemical — heptylaldehyd — in many cases caused extensive liquefaction of this tumor, followed in some instances by complete regression. Were it not for the fact that this effect is obtained only with a dosage rather close to the fatal dose, some hope of practical usefulness might be entertained. However, the interesting point I wish to emphasize is the selective action on the tumor.

The old gout remedy, colchicine, a phenanthrene derivative, is known to cause arrest in cell division. Recent attempts to utilize this action for the treatment of tumors have given negative results, because the drug is too toxic. Efforts are now being made to synthesize chemicals of closely related structure with the hope of finding a less toxic and effective compound.

In view of the startling development of the chemical treatment of certain bacterial and virus diseases, it would seem hopeful to pursue the search for chemical agents for cancer therapy. As in the case of most of the newer chemicals for the treatment of other diseases, the search for such chemicals for cancer has to be carried out by experimentation on animals, by making full use of any leads suggested by clinical observation.

In conclusion, I do not hesitate to predict that persistent, intelligent, and comprehensive effort along the lines mentioned in this lecture is bound to result in saving many thousands of lives.

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Effect of Foster Nursing on the Response of Mice to Estrogens

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Since the establishment of the fact that the tendency to develop mammary adenocarcinoma in mice can be segregated into certain strains, numerous investigators have attempted to elucidate the differences between mice which develop tumors of the breast and those which are relatively free of this neoplasm. These investigations were performed by comparing different inbred strains of mice, since the segregation of mammary carcinoma involved inbreeding of the animals and indeed was considered to be genetic. The result was that although various interesting differences were encountered, none of these differences was indubitably related to the presence or the absence of susceptibility to, and the development of mammary tumors.

The important studies of Murray and Little (*1*) and of Korteweg (*2*), on the extrachromosomal influences in the genesis of mammary carcinoma in mice, and of Bittner (*3–5*), on the effect of foster nursing on the occurrence of this neoplasm, make possible a better approach to the problem. Foster nursing of mice of strains which have a high incidence of mammary carcinoma (A, C3H, dba) by females of strains which are resistant to this tumor (C57 Black, C, Y), reduces the occurrence of the tumor in the fostered females. Conversely, foster nursing of mice of strains with a low incidence of mammary carcinoma (C, C57 Black) by mice which are susceptible to tumors of the breast (C3H, dba) increases markedly the incidence of mammary tumors in the fostered females (*5–7*). The same results can be reached in hybrid mice either by foster nursing or by the simpler process of reciprocal crossing. Thus, mice of identical genetic constitution, but with radically different incidences of mammary adenocarcinoma are obtained. Differences in these mice are much more likely to be related to the development of tumors of the breast than are differences elicited in the comparison of mice of widely divergent genetic constitutions. The method has been employed already by Van Gulik and Korteweg (*7*), who described interesting alterations in the architecture of the mammary gland in foster-nursed mice.

EFFECT ON VAGINAL RESPONSE

It has been established that hormonal stimulation, particularly that of estrogens, is one of the factors involved in the genesis of mammary tumors in mice.[1] No correlation has been found, however, between the manifestations of estrogenic stimulation, such as the duration of vaginal keratinization, number of estrus cycles and the normality of the cycles, and the susceptibility of various strains of mice to mammary carcinoma (*10–13*). On the other hand, Van Gulik and Korteweg (*14*) recently found that two strains of mice which are susceptible to tumors of the breast are less susceptible to estrogen, as determined by vaginal-smear assay, than mice of three low-mammary-cancer strains. The authors suggested that the females whose genital organs are least susceptible

[1] *For reviews, see Gardner* (*8*) *and Lacassagne* (*9*).

to estrogen produce the greatest quantity of the hormone under normal conditions, and that there may be a causal relationship between the degree of susceptibility of the genital organs to estrogen and the degree of disposition to tumors of the breast.

The implication that certain strains of mice have a high incidence of mammary carcinoma because they are relatively resistant to estrogen and must elaborate more of the hormone in order to reach normal estrus, is of obvious importance.

It was decided to test this theory by the use of foster-nursed mice. If the disposition to carcinoma of the breast is inversely related to the susceptibility of the genital organs to estrogens, foster nursing of mice resistant to mammary carcinoma by females which are susceptible to mammary tumors should increase the resistance of the fostered mice to estrogens by the vaginal-smear assay, and the fostering of mice susceptible to this tumor by females which are resistant to tumors of the breast should decrease their resistance to the hormone.

Experimental Procedure

Brother-sister mating of mice of strains C3H, C, and C57 Black, maintained at the National Cancer Institute, was started in May 1940. Litters were born between June 24 and July 13, 1940. Within 17 hours or less after birth, half of each C3H litter was transferred to a mother of strain C or C57 Black, and at the same time half of the litter of strain C or C57 Black was given to the C3H female. Thus, litter mates of strain C3H were obtained in which half were nursed by their own mothers and half were foster nursed by mothers of strain C or C57 Black; of the litters of C or C57 Black, half were nursed by their own mothers and half by C3H females.

The sexes were separated at weaning, and the mice were marked individually. The females were ovariectomized by the dorsal approach on August 22 to 27, 1940, when they were 6 to 9 weeks of age. The number of mice in which ovariectomy was successful as determined by preliminary vaginal smears, and which were used in the experiment, is given in table 1. The table also indicates the previously reported (*6*) incidences of mammary carcinoma in the normal and foster-nursed females of these strains.

The susceptibility of the mice to estrogens, as manifested by the vaginal reaction, was determined by the bioassay technique as outlined by Emmens (*15*). Crystalline 3-hydroxy-17-keto-Δ-1,3,5-estratriene (estrone),[2] dissolved in purified peanut oil, was the estrogen employed. On September 9, all mice were given a single subcutaneous injection of 0.1 microgram of estrone in 0.1 cc. of the oil as a "priming" dose. Assays were started 3 weeks later and were continued at fortnightly intervals on the same groups of mice. The estrone

[2] *Theelin, Parke-Davis.*

Table 1.—*Vaginal response to estrone in normal- and foster-nursed mice of strains C3H, C, and C57 Black*

Strain of mouse	Nursed by—	Incidence of mammary cancer	Number of mice	Number of positive vaginal smears, according to dose of estrone (in micrograms)						
				0.0125	0.025	0.0375	0.05	0.075	0.10	0.125
C3H	C3H	98+	31	.....	1	.....	5	16	19	21
C3H	C	25−	15	.....	1	.....	1	6	12	13
C3H	C57 Black	25−	17	.....	1	.....	2	8	11	12
C	C	5−	21	2	5	.....	13	.....	17	19
C	C	60	13	3	4	.....	9	.....	11	12
C57 Black	C57 Black	1−	23	.....	1	5	16	22	23	.....
C57 Black	C3H	10+	25	.....	2	9	19	25	25	.....

was always dissolved in 0.1 cc. of peanut oil; half of the dose was given on Monday and half on Tuesday.

Vaginal smears, obtained by a metal spatula, were taken at 24-hour intervals on 3 successive days after the second injection of estrone. The smears were stained with methylene blue. A reaction was considered positive when one or more of the smears contained at least 50 percent of cornified epithelial cells and no more than extremely rare leukocytes.

Results

The results, which are summarized in table 1, were tested statistically by the procedures outlined by Bliss (*16*).[3]

In figure 1, the results in mice of strain C3H are plotted as probits against log-dose, and the calculated regression lines are drawn through the observed points. There is no difference in the regression lines, in either the slopes or the positions, for the normal C3H mice as compared with those foster nursed by C mice ($P=0.77$ and $P>0.99$, respectively), or for the normal C3H mice as compared with those foster nursed by C57 Black females ($P=0.78$ and $P>0.99$, respectively). Thus, although foster nursing of C3H mice by mice of strains C or C57 Black reduces the incidence of mammary carcinoma from practically 100 percent to 25 percent or less (*17*), it does not affect the resistance of the mice to estrone as far as the vaginal reaction is concerned.

Figure 2 presents similar negative results for mice of strains C and C57 Black.

[3] *We are indebted to Dr. W. Ray Bryan for the calculations.*

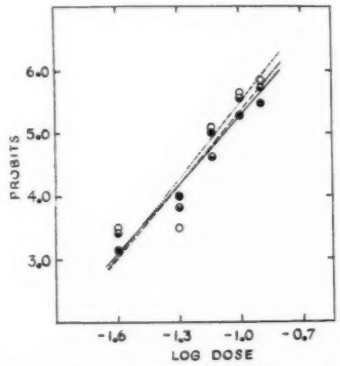

FIGURE 1.—Dose-response curves for vaginal response to estrone in normal and in foster-nursed C3H spayed females, showing regression of percentage positive smears (in probits) on log-dose.

Solid circles and solid line, normal C3H.
Half circles and dash line, C3H fostered by C57 Black.
Open circles and dotted line, C3H fostered by C.

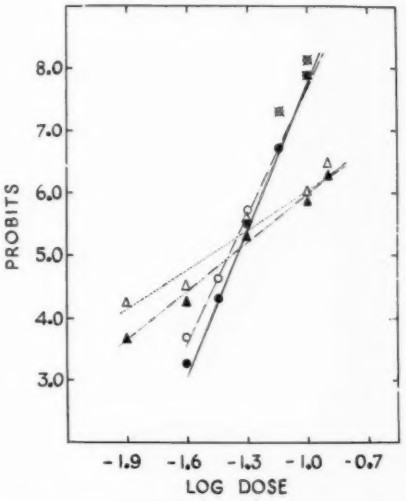

FIGURE 2.—Dose-response curves for vaginal response to estrone in normal and foster-nursed C57 Black and C spayed females, showing regression of percentage positive smears (in probits) on log-dose.

Solid circles and solid line, normal C57 Black.
Open circles and dot-dash line, C57 fostered by C3H.
Solid triangles and dots-dash line, normal C.
Open triangles and dotted line, C fostered by C3H.
Crossed circles indicated estimated values for 100-percent responses.

Strain C mice which were foster nursed by C3H females do not differ in their response to estrone as compared with normal controls ($P=0.59$ for the discrepancy in slope, and $P=0.37$ for the discrepancy in position of the regression lines), although this procedure increases the incidence of mammary tumors from less than 5 percent to over 50 percent (*6*). Normal C57 Black females have a negligible incidence of mammary tumors, but when foster nursed by C3H mice the incidence is about 10 percent (*6*); the vaginal reaction to estrone, however, remains the same ($P=0.58$ for the discrepancy in slope, and $P=0.40$ for the discrepancy in position of the regression lines).

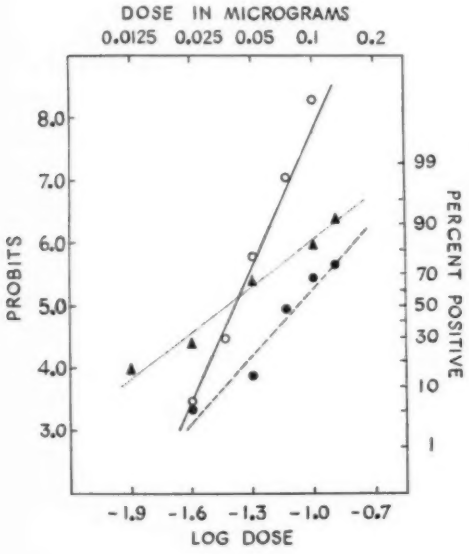

FIGURE 3.—Dose-response curves for vaginal response to estrone in mice of strain C3H, C, and C57 Black, showing regression of percentage positive smears (in probits) on log-dose.

Closed circles and dash line, C3H. The equation of the regression line, when y is response in probits and x is log-dose, is $y=9.098+3.765x$.

Open circles and solid line, C57 Black. Regression equation is $y=15.086+7.319x$.

Closed triangles and dotted line, C. Regression equation is $y=8.471+2.441x$.

Since there is no significant discrepancy in the results obtained with normal and foster-nursed animals of the three strains, the data of the two groups are combined in figure 3 to give a better estimate of the strain response in each case. When the responses of the three strains are compared on this basis, significant differences are obtained. The slope of the regression line is steepest for the C57 Black strain, indicating that the variability among individual animals is less in this strain than in the other two. The value of P for the discrepancy in slope is less than 0.01 when compared with the slopes of either C or C3H. When the C and C3H strain responses are compared, the discrepancy in slope is statistically significant ($P=0.019$). The three strains of mice, therefore, differ significantly in the degree of variability shown by individual test animals in their vaginal responses to estrone.

The strain susceptibilities to estrone may be compared on the basis of the quantity of the hormone required to produce a vaginal reaction in 50 percent of the test animals; i. e., the median effective dose or ED_{50} (*18*). The ED_{50} for the vaginal reaction to estrone in C3H mice is 0.08 ± 0.004 microgram. The ED_{50} for C strain animals is 0.038 ± 0.005 microgram, and for C57 Black mice it is 0.042 ± 0.0015 microgram. The difference between strains C and C57 Black is not significant ($P=0.45$), but the difference between either of these two strains and C3H mice is highly significant ($P<0.01$). Thus, mice of strain C3H are approximately twice as resistant to estrone, as measured by the arithmetic value of ED_{50} for the vaginal response, as mice of strains C or C57 Black.

Discussion

The study shows that there is a marked difference in the vaginal response to estrone in the three strains of mice used. The susceptibility to estrone is inversely related to the susceptibility of the three strains, under normal conditions, to the incidence of mammary carcinoma; i. e., mice of two strains which are resistant to the development of the neoplasm (C and C57 Black) are twice as susceptible to estrone as mice of a strain which has a high incidence of tumors of the breast (C3H).

Foster nursing, however, does not alter the susceptibility of the mice to estrone, as manifested by the vaginal reaction, although it alters radically the degree of disposition to carcinoma of the breast. The differences in the vaginal response to estrone, therefore, are strain peculiarities and are not causally related to the genesis of mammary adenocarcinoma.

EFFECT ON INDUCTION OF MAMMARY TUMORS

Twombly (*19*) has indicated that foster nursing influences the response of male mice to estrogens as far as the induction of mammary carcinoma is concerned. Of 27 male mice of strain C57 Black which had been fostered by R III (high mammary tumor) strain females and which were implanted with crystals of estrone at 10 days of age, 9 developed mammary tumors in 7.5 to 11 months. This incidence is much higher than can be obtained in normal males of this strain. Bittner (*5*) recently reported similar results. After the implantation of a crystal of estrone or estradiol benzoate, 6 of 19 normal strain A males and females developed mammary tumors in an average of 185 to 190 days, whereas no tumors were induced in 43 strain A mice which were foster nursed by C57 Black females. Three tumors were induced in 12 normal C3H (JAX) mice, whereas 18 C3H animals which were foster nursed by C57 Black mice were negative.

Our studies, which are still in progress, are in agreement with those of Twombly (*19*) and of Bittner (*5*). Data of the preliminary experiments are sufficiently impressive to be noted here.

Experimental Procedure

Male mice of strain C3H and C, half of which were nursed by their own mothers and half of which were foster nursed by C57 Black mice and C3H females, respectively, were obtained. A group of strain C females, normal and foster nursed by C3H mice, was also used.

The estrogen employed was 4,4′-dihydroxy-α,β-diethylstilbene (stilbestrol [4]) given either in 20 weekly subcutaneous injections of 0.2 mg. dissolved in 0.1 cc. of sesame oil, or as a single subcutaneous implantation of a cholesterol pellet containing 10 to 50 percent of the compound and weighing 5 to 8 mg.[5]

Results

The results up to 10 or 12 months are presented in table 2. Whereas over 60 percent of the normal C3H males have developed mammary tumors, only 1 tumor has appeared in the C3H males which were foster nursed by C57 Black females. This C3H male had remained with its own mother for about 17 hours after birth (*17*). Four mammary tumors have appeared among 13 strain C males which were foster nursed by C3H females, and none in the normal males. Seventy percent of the female mice of strain C which

[4] *Diethylstilbestrol, Merck.*

[5] *Shimkin, M. B., and Grady, H. G.: Data to be published.*

TABLE 2.—*Effect of foster nursing on the induction of mammary carcinoma with stilbestrol, in strains C3H and C male and strain C female mice*

Strain of mice	Sex	Nursed by—	Number of mice	Mammary tumors, in months after stilbestrol administration									Number of tumors
				4	5	6	7	8	9	10	11	12	
				No.	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	
C3H	M	C3H	22			1	3	5	1	4	1		15
C3H	M	C57 Black	31						1				1
C	M	C	20										0
C	M	C3H	13				1	2	1				4
C	F	C	34					1					1
C	F	C3H	20	1	6	2	1	2	2				14

were foster nursed by C3H mice have developed mammary carcinoma by 9 months, whereas the incidence among C females nursed by their own mothers is under 5 percent.

Confirmatory results from three different sources, therefore, permit the conclusion that foster nursing radically affects the ability of the mouse, of either sex, to develop tumors of the breast following massive administrations of estrogen.

DISCUSSION

As Lacassagne (*20*) summarized the problem:

> Carcinoma can occur only in a mammary gland which has undergone a certain degree of development. The development of the mammary gland is dependent upon the estrogenic and pituitary hormones. But whether these stimulating factors play a direct role in the cancerization process, or only produce the anatomical development of the mammary gland sufficient to allow the cancer process to manifest itself is still undetermined.

The marked influence of the milk factor upon the genesis of mammary tumors, both those of spontaneous origin and those induced with estrogens, makes it evident that estrogens merely prepare a suitable substratum in the breast of the mouse for the action of another factor, presumably transmitted through the mother's milk. Since the incidence of mammary carcinoma in C3H females foster nursed by C57 Black mice was found by Andervont (*17*) to be directly proportional to the number of hours the young remain with their mothers, this factor is a carcinogenic agent (or agents) derived from mice of high-mammary-tumor strains rather than an anti-carcinogenic factor derived from mice of low-mammary-cancer strains. Bittner (*5*) has reported that the factor can be transmitted to month-old strain A females by the oral administration of milk obtained from lactating females of a cancerous stock. There is no indication at the present time as to what the nature of this factor (or factors) may be, and it remains one of the most engrossing problems of experimental oncology.[6]

Bittner (*4*) has stated that three factors must be involved in the genesis of mammary tumors in mice: the genetic, the hormonal, and the milk. Murray and Little (*1*) have shown that the genetic factors are only one-tenth as important as the extrachromosomal influences. The present studies and those of Twombly (*19*) and of Bittner (*5*) indicate that the hormonal factors also must be relegated to a secondary position in the etiology of mammary adenocarcinoma in mice.

CONCLUSION

Foster nursing exerts no influence upon the vaginal reaction to estrone in mice of strains C3H, C, or C57 Black.

[6] *No growth is obtained in cultures of milk from C3H and from C57 Black mice on blood agar and on developing hen and duck eggs. (Shimkin, M. B.: Unpublished data).*

Foster nursing of mice of a strain with a high incidence of mammary carcinoma (C3H) by mice with a low incidence of this neoplasm (C57 Black), reduces their susceptibility to induction of mammary carcinoma following massive administrations of estrogen. Foster nursing of mice of a strain with a low incidence of mammary carcinoma (C) by mice with a high incidence (C3H) increases their susceptibility to mammary-tumor induction with estrogens.

Acknowledgement is made of the technical assistance of Mr. Walter F. Gately.

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Studies on the Purification and Properties of the Rabbit-Papilloma-Virus-Protein[1]

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[1] *This investigation was carried on at the Department of Surgery, Duke University School of Medicine, and was aided in part by a grant from the National Cancer Institute. The several studies which are brought together in the present article for a unified presentation of the findings with the papilloma virus were published originally as a series of separate reports appearing in the Journal of Infectious Diseases and in the Journal of the National Cancer Institute.*

INTRODUCTION

The rabbit papilloma (Shope), or infectious papillomatosis, is an endemic disease of western cottontail rabbits characterized by the presence of warty epidermal growths. The condition, though long known in the region where it occurred, was studied first by Shope (*1*) in 1933. This investigation revealed the disease to be infectious and the etiological agent to possess characters classifying it as a typical member of the group of filterable viruses.

The newly characterized disease quickly aroused numerous interests. In addition to obvious possibilities for investigation of the condition as a virus disease were those related to the biological aspects of the growth *per se*. Studies in the latter field showed that the papillomatous process in both cottontail and domestic rabbits exhibits in many respects the behavior of mammalian tumors (*2–4*). The interpretation and the significance of these findings were subsequently greatly emphasized by the discovery that the papillomatous growth progresses with time to typical destructive and metastasizing carcinoma, frequently in domestic rabbits (*5*) and occasionally in cottontail rabbits (*6*). Further, by means of appropriate preparation, the response of the epidermal cells to the papilloma virus can be altered (*7*), so that carcinoma develops quickly without the usual latent period observed when virus is applied to cells injured simply. These and related investigations have placed the rabbit papilloma and the carcinomas deriving from it in the category of mammalian tumors, differing from them chiefly with respect to the known etiology.

For many years a most interesting phase of tumor research has been the study of a small group of virus-induced growths fulfilling in many respects the criteria of neoplasms in general. Chief among them are the leukosis of fowls and the Rous chicken sarcoma. To these has now been added the rabbit papilloma, which, however, occupies a unique position as the only established virus-induced mammalian neoplasm. Studies of these diseases as tumors have added greatly to the knowledge of neoplasms in general. In addition to the opportunities offered by this line of investigation is another which is of at least equal importance and promise for the tumor problem. This is concerned with the possibility presented for the direct study of the nature and properties of definite, specific tumor-causing agents, namely, the viruses responsible for the respective growths. This phase of the problem recently has received much attention. Developed chiefly in association with work on plant viruses (*8*), methods are now at hand for direct approach to questions of the nature and properties of the tumor-causing, as well as other viruses. Use of these procedures has been made in the study of the agents responsible for the Rous chicken sarcoma (*9*), the rabbit papilloma (*10*), and leukosis of fowls (*11*). The measure of success attained in a relatively brief period not only has resulted in the accumulation of much useful and significant information of immediate interest but also has demonstrated the efficacy of the methods applied, and has indicated a great degree of promise offered by persistent efforts in this direction.

For several years work of this sort has been under way with the rabbit-papilloma virus, and the findings have been presented from time to time. In many respects choice of this material has proved fortunate. Owing to factors concerned both with the nature of the virus and that of the material from which it can be derived, its isolation and purification are relatively simple. Further, the properties of the purified material are such that unusual opportunities are offered for quantitative

investigations on its biological behavior. The work thus far has been concerned to a relatively small extent with physical and chemical studies, being devoted principally to investigations on the biological properties and behavior of the purified virus. In the present paper are collected the principal findings thus far encountered.

PURIFICATION AND GENERAL PROPERTIES OF THE PAPILLOMA VIRUS

Extracts of warts occurring naturally in western cottontail rabbits are ordinarily highly infectious though some exhibit little infectivity (*1*). Growths induced experimentally in cottontail rabbits yield extracts usually of low-grade infectivity, while those of warts induced similarly in domestic rabbits are rarely infectious (*12*). The methods successful in the isolation and purification of the virus have been applied to material from both cottontail and domestic rabbits (*13*). Significant quantities of virus have been obtained only from highly infectious cottontail-rabbit-wart extracts, and the yield of it has been roughly proportional to the infectivity of the extracts.

Method of Purification

The procedure for purifying the papilloma virus has consisted essentially in simple ultracentrifugal fractionation of extracts containing the virus. For this a replica of the air-driven quantity ultracentrifuge described by Wyckoff and Lagsdin (*14*, *15*) has been used. The reason for the fractionation sequence employed and the interpretation of the findings are clarified by consideration of the nature of the material involved.

The rabbit papilloma occurs naturally and can be induced experimentally only in the skin. Arising from the basal cells of the epidermis, the growth increases in size through multiplication of epidermal cells, which rapidly become keratinized, and through increases of attendant connective tissue supporting structures. In contrast with the fate of epidermal cells under normal conditions, the keratinized papilloma cells are not desquamated as the growth increases in size. The superficial areas usually become dry and elastic or sometimes horny and enclose a moist keratinized mass of amorphous papilloma cells into which thin papillae of living cells extend from the base where the mass is seated. For the most part, then, the growths consist of a structureless, lifeless mass of cell debris comprised of particles of pigment, keratin, and insoluble denatured protein. Extracts of such material are poor in the usual viscous tissue protein which is difficult to eliminate in the purification process. Experience has shown that the virus in growths carrying it is distributed throughout the mass, to some extent even in the superficial dried regions.

The experiments reported here, except in specified instances, were made with warts found on rabbits trapped in Kansas. Growths were harvested and placed in 8-ounce bottles containing a mixture of equal parts glycerin and 0.9 percent NaCl solution. The bottles were kept in the field until filled with warts so that each nearly always contained growths from several rabbits. The bottles were shipped to the laboratory and stored at 5°–8° C. Under these storage conditions the virus activity of the growths is maintained indefinitely. The growths have been used after periods up to 3 years. The results obtained with four batches of warts produced by tattooing virus into numerous spots in the skin of captive cottontail rabbits are also described in this paper. These growths had been in glycerin storage for 11 months.

Freed of connective tissue and hair, warts were ground to a smooth paste with sand and extracted, for about 12 hours, in 10–20 times their weight of 0.9 percent NaCl solution. Extractions were made also with solutions containing other concentrations of NaCl; less virus was obtained where the salt content exceeded 2 percent, and still less when distilled water was employed. Sand and gross tissue particles were removed from extracts by twice-repeated spinning in the ordinary horizontal or angle centrifuge. A single extraction removed more than 90 percent of the virus.

The crude extract was distributed in 15.5-cc. volumes in 18.0-cc. collodion tubes which were then placed in the 8-tube ultracentrifuge rotor chilled with cracked ice. The rotor was sealed and spun rapidly up to 9,000 r. p. m. (about 7,000 times gravity) and quickly stopped (the run-up-and-down). The sediment from this operation was discarded, and the supernatant fluid, poured into fresh tubes, was ultracentrifuged in a field of about 40,000 times gravity for 1 hour. The supernatant fluid from this first ultracentrifugation was discarded, and the tubes containing the sedimented pellets were inverted for a few minutes to permit the excess fluid to drain away. The pellets were then taken up in 0.05 M phosphate buffer pH 6.5 with the aid of fine-tipped pipettes. Ordinarily the volume of solution employed at this point is 31.0 cc. for eight pellets, just enough for two of the collodion ultracentrifuge tubes. The suspension was cleared of aggregated material by a run-up-and-down as before. The supernatant fluid was then spun in two clean tubes at 40,000 times gravity for 1 hour. The two pellets were suspended in 15.5 cc. of buffer solution, and the volume was maintained for the remainder of the procedure except for the final solution of the pellet. The sequence of alternate high- and low-speed centrifugation was continued through five cycles, and the last pellet dissolved in 1.0–5.0 cc. of buffer solution pH 6.5 depending on its size. The final solution was spun in a small collodion tube at full speed for 20 minutes in the angle centrifuge.

Crude extracts of papillomas are amber in color and slightly turbid after low-speed centrifugation. The preliminary run-up-and-down in the ultracentrifuge removes much suspended material leaving a liquid which, though still amber, is macroscopically almost clear. Pellets obtained from the first ultracentrifugation are opaque and brown, consisting primarily of aggregated colloidal material including finely divided melanin. They are friable and suspend easily without adhering to either the tube or pipette tip. Such suspensions, after vigorous pipetting for 10–20 minutes, have a whitish tinge. Most of the colored material, present in relatively large floclike particles, is removed by the second run-up-and-down to yield a grayish-white and opalescent suspension. Except for a small opaque core, the pellets from the second high-speed ultracentrifugation are almost clear. These and subsequent pellets, in contrast with the earlier ones, are sticky, can be drawn into thin strands with the pipette tip, and are adherent, not friable. Concentration lines are seen around them as they go into solution. After the second ultracentrifugation, relatively little material is lost in the run-up-and-down. Pellets from the third and following ultracentrifugations are usually macroscopically clear and, though sometimes as much as 7.5 mm. in diameter, are scarcely visible when covered with fluid.

The effects of the fractionation process on infectivity of the extracts are illustrated

in table 1 and figure 1. In table 1 are compared the infectivities of the various fractions obtained in a typical experiment. Though the tests were crude, it is apparent that infectivity was carried through the process in association with the material sedimented by full ultracentrifugation. The efficacy of ultracentrifugation in the sedimentation of the infectious material of a crude extract under the prescribed conditions is shown quantitatively in figure 1. In this experiment is shown the residual infectivity of the supernatant fluid of the extract spun at 40,000 times gravity for various intervals. The ultracentrifugal process results in the sedimentation and concentration of most of the virus within the period of 1 hour.

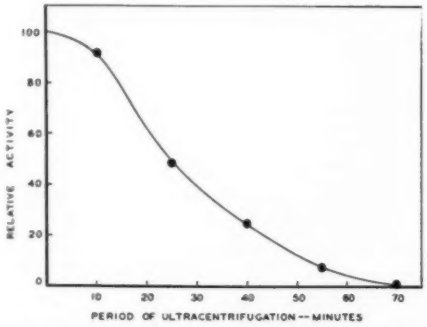

FIGURE 1.—Sedimentation of infectivity from crude papilloma extract in a gravitational field of 40,000 g. (Infectivity measurements made by incubation-period analysis. See text.)

ULTRACENTRIFUGAL SEDIMENTATION PATTERN

Development of the fractionation sequence and judgment of its success were directed primarily by examination of the concentrates with the analytical ultracentrifuge (*14–16*). By this means information was obtained relative to the physical state of the material. For these studies an air-driven instrument was employed which utilized the absorption method for photography with ultraviolet light of 2300–2700 A°. Cells containing the concentrate solution for the examination were 2 mm. thick.

The crude extracts of papillomas contain in solution small quantities of tissue protein, and in suspension insoluble particles of pigment and denatured protein of greatly varied size. When the crude extract is examined in the analytical ultracentrifuge at progressively increasing speeds, there is seen to sediment a light-absorbing material of great inhomogeneity, evident in the gradual diminution of light absorption from the bottom of the cell upwards. When the speed of the centrifuge reaches a level where the centrifugal force becomes comparable with that

TABLE 1.—*Infectivity of ultracentrifugal fractions of an extract of cottontail rabbit papillomas*

[Papillomas[1] in test rabbits after inoculation]

Material	Dilutions[2]											
	1-200				1-2,000				1-20,000			
	Rabbit				Rabbit				Rabbit			
	1	2	3	4	1	2	3	4	1	2	3	4
Crude extract	+++	++++	+++±	++++	++	++	++	++++	+	+	±	+
Supernatant first run-up-and-down	+++	++++	+++	++++	++	+++	+++	++++	±	+±	±	++
Pellet first run-up-and-down	+	+++	+±	+++	+	+	+	+	0	0	0	±
Supernatant first ultracentrifugation	0	0	0	±	0	0	0	0	0	0	0	0
Pellet first ultracentrifugation	++++	+++	+++	++++	+	++	++	++	±	+	±	+
Pellet third ultracentrifugation	++	+++	+++	++++	+	+++	++	+±	0	±	±	±

[1] 0=Negative; ±= 1–6 papillomas; ++++= confluent papillomas; other symbols indicate papillomas of intermediate size.
[2] Dilutions based on weight of papilomas extracted.

exerted in the purification process, all of this inhomogeneous substance has been eliminated from suspension. The solution which remains likewise absorbs light because of the presence of the tissue protein. Because of the smaller molecular size of this protein, no sedimentation of it occurs in the centrifugal field of the magnitude described. Photographs thus show an even absorption of light over the area of the cell, and the substance or substances responsible for the absorption are termed for convenience "unsedimentable materials." The term is entirely relative, for in ultracentrifugal fields of appropriate force the low-molecular-weight protein is likewise sedimented.

Results of another character are found when the concentrates of highly infectious wart extracts are studied. The difference is evident in some instances with solutions of the first ultracentrifugal pellet. Here, there is frequently present a definite, or even rather sharp, boundary unrelated either to the inhomogeneous particulate material or to the small amount of low-molecular-weight tissue protein carried over with the pellets and from the walls of the tubes. In solutions of the second ultracentrifugal pellet, the boundary becomes more prominent, though some of the unsedimentable protein may still be present. After three or more cycles, only the pattern characteristic of the purified concentrate is observed. The sedimentation pattern of a typical preparation is shown in figure 2. This consists of a principal boundary which has the sedimentation constant $s_{20°}$=ca. 260×10^{-13} cm. sec.$^{-1}$ dynes^{-1}. Frequently there is present also a faint secondary boundary with $s_{20°}$= ca. 375×10^{-13} cm. sec.$^{-1}$ dynes^{-1}. Concentrates from good warts carefully handled in the cold give single sharp boundaries; whereas if they are treated less carefully, the boundaries are shaded or diffuse. These results show that (1) all unsedimentable tissue protein demonstrable by the method has been eliminated in the process, (2) detectable amounts of the inhomogeneous colloidal material initially present have been discarded, and (3) the final concentrate exists primarily as a single entity of the homogeneity, with respect to ultracentrifugal sedimentation, of a single molecular species.

FIGURE 2.—Sedimentation diagram of papilloma-virus protein. $S_{20°}=260\times10^{-13}$ cm. sec.$^{-1}$ dynes^{-1}.

General Chemical and Physical Properties

Purified virus concentrates give positive reactions to the usual tests for protein and are precipitated quantitatively by hot 5 percent trichloracetic acid. Molisch tests on dialyzed solutions containing 2.6 mg. protein were negative. The protein is coagulated at 65°–67° C. The results of elementary chemical analysis of papilloma-virus protein which was ultracentrifuged five times, in percentages of dry weight are: C, 49.56; H, 7.15; N (Dumas), 14.99; N[2] (Kjeidahl), 14.5; P, 0.944; S, 2.2; ash, 2.5; and acetone-soluble material, 3.2. The concentrate for this analysis was dialyzed against distilled water overnight, precipitated with acetone, and washed with ether. It was dried *in vacuo* over P_2O_5 at 50°. The acetone and ether were evaporated to give the estimate of acetone-ether-soluble material indicated. These results show that the concentrates are

[2] *Material for this analysis was precipitated with trichloracetic acid and dried over P_2O_5.*

essentially protein in nature without conjugated polysaccharide. The significance of the small fat content is questionable; the value may indicate the presence of fat as an integral part of the material but may represent only a residual component carried over from the original wart tissue.

The protein is soluble in 0.05 M phosphate buffer or NaCl solution. Precipitation occurs when the salt content is lowered to 0.005M, and the protein with the infectivity can be sedimented in the angle centrifuge. With increase in salt concentration, the precipitate dissolves, and the solution possesses the initial infectivity. The isoelectric point of the protein, determined with the Northrop-Kunitz cell, lies at about pH 5.0, and here the protein precipitates rapidly, carrying the infectivity with it. When the pH is lowered to 4.2 or raised to about 6.0, the protein redissolves, and the solution is fully infectious.

Influence of Hydrogen-Ion Activity on Infectivity and Molecular Stability

The pH stability of infectivity determined by titration and that of the protein examined with the analytical ultracentrifuge (*17*) are shown in figure 3. The stability range of infectivity is between pH 3.0 and 7.5 with the optimum in the region of the isoelectric point. Infectivity is destroyed immediately at pH 2.5 to 3.0 and, as shown by the analytical ultracentrifuge, degradation of the protein occurs at pH 2.8 to yield noninfectious disintegration products consisting of light, unsedimentable material and a homogeneous macromolecular protein with $s_{20°}$=ca. 200×10^{-13} cm. sec.$^{-1}$ dynes^{-1}. In alkaline solution, infectivity diminishes more slowly with increase in pH than after comparable decreases in pH on the acid side. Decrease in infectivity resulting from the action of alkali, however, is not accompanied by sudden change in the sedimentation constant. Noninfectious solutions near pH 10 gave the usual constant; disintegration occurred slightly above pH 10 to yield unsedimentable material and a noninfectious homogeneous product with $s_{20°}$=ca. 129×10^{-13} cm. sec.$^{-1}$ dynes^{-1}, while at pH 10.7 further splitting gave a lighter derivative with $s_{20°}$=ca. 30×10^{-13} cm. sec.$^{-1}$ dynes^{-1}.

Occurrence and Yield of the Protein

The yield of papilloma protein from different batches of cottontail rabbits is widely variable. The range observed has extended from 1 mg. of protein per gram of highly infectious naturally occurring warts to 0.008 mg. per gram of warts of low infectivity obtained by experimental inoculation of cottontail rabbits. No trace of the characteristic concentrate has been obtained from noninfectious domestic-rabbit warts despite repeated examinations of both fresh and glycerolated growths. In such experiments the ultracentrifugal fractions which should have contained the protein were water clear and completely devoid of it. It should be emphasized that in noninfectious extracts, either from cottontail- or domestic-rabbit growths, there is no material yielding any evidence

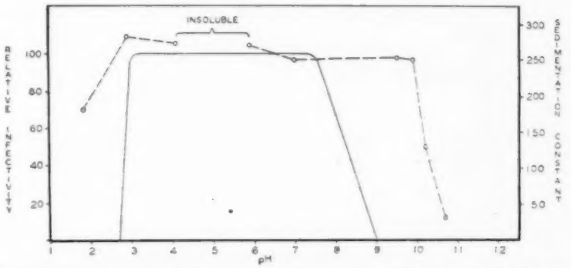

FIGURE 3.—pH stability of papilloma-virus protein determined by infectivity (solid line) and sedimentation constant (broken line).

of homogeneity in the ultracentrifugal range used for purifying the virus protein. In this respect interpretations of the findings are not clouded by the presence of complicating normal-tissue components sedimenting with the virus as in the instances of the purification of the Rous sarcoma virus (*9*) and the equine encephalomyelitis virus (*18*). The early experiments made to measure the relation between the infectivity of the original extracts and the yield of protein were too crude to make possible an unequivocal statement of the degree of parallelism. Experiments made recently with methods suitable for the quantitative estimation of virus infectivity show, however, that infectivity of the purified material is directly proportional to protein mass. The finding of a homogeneous material only in the presence of virus manifestation lends great emphasis to the probable identity of the virus with the protein concentrate. This was substantiated by other experiments in the series showing the general dependence of virus integrity on the physical and chemical state of the protein concentrate.

ESTIMATION OF PURIFIED PAPILLOMA-VIRUS INFECTIVITY

An obviously important phase of the study of viruses in general is that concerned with the quantitative determination of infectivity or the measure of that capacity of the agents which enables them to become established and to increase in susceptible host tissue. Values obtained in quantitative studies on infectivity may be relative or absolute. In the former instance the respective infectious capacities of two preparations may be compared in the same host as, for example, when a treated virus material is inoculated in a group of animals which likewise receive an untreated or control virus preparation. The values observed, though perhaps accurate, have quantitative meaning only with respect to the one experiment, and direct comparison of the results in one set of experiments with those of another can be made only if some means is at hand for maintaining a "standard" control. With viruses this is difficult because of their lability. Furthermore, even if a standard control preparation were available, the usefulness of data obtainable with it would still be sharply limited. It should be emphasized that a concept of quantitative infectivity involves consideration of the relation between virus effect and virus mass. A value describing this relation has meaning that is absolute only within the limits of the methods for determining effect and mass. Actually, knowledge of the relation between effect and mass is still not enough. The various viruses are definite physical entities which differ in size, specific gravity, etc., and thus with respect to the number of virus particles per unit mass. With present knowledge, then, the problem of estimating absolute virus infectivity consists in a consideration not only of mass but also of the number of virus particles concerned.

Studies were made on the purified papilloma-virus protein from this point of view. The chief objective to begin with was to see whether or not the purified product would exhibit a uniform behavior with respect to infectivity and, if so, to determine the relation between effect and mass. Experience soon showed that the procedures ordinarily employed for papilloma-virus titration were wholly inadequate to provide data suitable for quantitative interpretation. It then became necessary to direct attention to the development of a procedure for infectivity estimation.

Relation of Incubation Period to Virus Mass

The response of domestic rabbits to the purified papilloma virus is highly variable as illustrated in table 2 in the results obtained by the inoculation of twofold dilutions of the protein in the 2 sides of each of 12 rabbits. While a completely refractory animal has not been observed, some rabbits develop small abortive lesions only with relatively concentrated materials and in some animals all warts frequently retrogress early. With high dilutions the induced papillomas are small, widely scattered, and they frequently retrogress before an unequivocal diagnosis can be made. The usual method of papilloma-virus titration consists in the inoculation of graded dilutions of it into domestic rabbits, and infectivity is described in terms of titer or the highest dilution causing lesions. Preliminary studies of papilloma-protein infectivity made with this end-titer method yielded only discouraging results. The findings were then examined with the statistical procedure suggested for vaccinia virus by Parker and Rivers (*19*). This method, of a sort commonly used for numerous biological assays, consists in the statistical study of the frequency distribution of test-animal responses to inocula of graded virus content. As the most stable point of reference in expressing degree of infectivity, the 50-percent point is chosen. This corresponds to that virus inoculum inducing lesions at half the number of sites inoculated and no lesions in the remainder of the spots. It was soon found that this procedure was but little more useful than end-point titer under the practical conditions for routine quantitative work with the papilloma virus.

Further analysis of the findings, however, revealed a relation which has furnished a means for the quantitative estimation of papilloma-virus infectivity with a high degree of expected accuracy. It has been

Table 2.—*Occurrence of lesions from a series of identical inocula in the left and right sides of 12 test rabbits* [1]

Rabbit No.	Side	pI															
		5.0	5.3	5.6	5.9	6.2	6.5	6.8	7.1	7.4	7.7	8.0	8.3	8.6	8.9	9.2	9.5
23	Left	+	Not inoculated				+				+						
	Right	+					+			+							
22	Left	+					+	+	+	+	+	+					
	Right	+					+	+	+	+	+						
21	Left	+					+	+	+	+		+					
	Right	+					+	+	+	+	+	+					
16	Left	+					+	+	+	+	+	+					
	Right	+					+	+	+	+	+						
24	Left	+					+	+	+	+	+		+				
	Right	+					+	+	+	+	+	+					
19	Left	+					+	+		+	+		+				
	Right	+					+	+	+	+	+	+	+				
18	Left	+					+	+	+	+	+	+	+				
	Right	+					+	+	+	+	+	+			+		
20	Left	+					+	+	+	+	+	+	+				
	Right	+					+	+	+	+	+	+	+	+			
15	Left	+					+	+	+	+	+	+	+				
	Right	+					+	+	+	+	+	+	+	+	+		
13	Left	+					+	+	+	+	+	+	+	+			
	Right	+					+	+	+	+	+	+	+	+			
14	Left	+					+	+	+	+	+	+	+				
	Right	+					+	+	+	+	+	+	+		+	+	+
17	Left	+					+	+	+	+	+	+	+		+	+	
	Right	+					+	+	+	+	+	+	+	+	+	+	+

[1] Arranged in order of response.

a common observation (*1*) that the inoculation of crude papilloma-virus extracts leads to the induction of growths after varying periods of time. It has been noted that this time interval, the incubation period, bears a relation to the amount of virus used. In the present experiments with the purified virus this relation has been found to be quantitative, and a basis has been established for the estimation of infectivity by means of it (*20*).

For quantitative studies on the infectivity associated with the protein, it is necessary first to have a method for the estimation of protein mass. The purified protein contains 15 percent nitrogen, and this property has been made the basis for protein assay. For this the photoelectric colorimeter (*21*) has been used for the determination of protein by turbidity measurements. Micro-Kjeldahl analyses were made on several batches of protein, and aliquot parts of the same materials were diluted with 0.9 percent NaCl solution and precipitated with hot 5 percent trichloracetic acid. Measurements of the turbidity developed were made in the photoelectric colorimeter using a standard consisting of a 1.0-percent solution of copper sulphate ($CuSO_4$. 5 H_2O) in 0.25 M sulphuric acid. By relating the results of turbidity measurements with those obtained in nitrogen analysis, a method was available for assays involving practical amounts of the protein.

The experimental technique for infectivity tests was the same as that usually employed for the papilloma virus. The animals were domestic rabbits of brown, gray, or agouti breed. Small areas about 1 inch square were shaved on the sides and abdomen, these regions accommodating about 24 spots. For the inoculations the areas were scarified with fine sandpaper, and into them the desired inoculum of 0.1-cc. volume was rubbed with the rounded end of a small sterile test tube. The animal was held quiet until the sites were dry; small squares of sterile gauze were placed carefully over each spot and fixed with adhesive tape. A large square of sterile gauze was then placed over the whole area and fastened over the back with tape. The whole was protected with a jacket retainer with holes for the front legs and tie strips for fastening over the back. Routinely the coverings were removed on the seventh or eighth day following inoculation. The areas were examined thereafter at intervals of 2 or 3 days.

Protein solutions prepared in the usual way were diluted with 0.9 percent NaCl solution to contain 0.1 mg. protein per 1.0 cc. This solution constituted the standard or stock, and inocula were prepared by consecutive twofold dilution with 0.9 percent NaCl solution. For the individual inoculations, the volume of protein solution was always 0.1 cc., and protein concentration is given as grams per 0.1 cc. The expression initially used in referring specifically to inocula was "log grams protein per inoculum." The term proved cumbersome, and it was suggested to us that the expression "log grams protein per inoculum" be treated in much the same way as hydrogen-ion concentration by employing the symbol pI to represent the logarithm of the reciprocal of the number of grams of protein per inoculum. Thus, if a given inoculum contains $10^{-5.3}$ gm. protein, this value is indicated by pI 5.3. In order to provide in advance a means for correlating the present results with those of studies on unpurified virus, certain of the findings and calculations have been expressed in terms of dilution (log dilution) as well as protein value. Consistency is maintained by the use of the symbol pD to designate the log of the reciprocal of the dilution. The dilution 1:2 or dilution $10^{-0.301}$ in this way becomes

simply pD 0.301. It should be noted that whereas pI is an expression representing absolute quantity, pD represents relative values only.

The relation of incubation period to protein amount is shown in the combined results of 4 separate experiments. In each experiment a different batch of protein was employed, and in each instance the pI of the lowest dilution was the same, namely 5.0. A total of 497 inoculations in 30 rabbits resulted in the induction of lesions in 376 areas. The combined data of the 4 experiments have been analyzed statistically to learn the nature and degree of correlation between incubation period and quantity of protein inoculated, or pI. The data have been classified according to pI, and observations on the incubation period have been grouped in class intervals of 2 days. The relation is shown in table 3. The data show a high correlation of incubation period with pI, which is confirmed by the high value of the correlation coefficient, $r=0.849\pm0.015$. The high value of the coefficient and the relatively small value of its standard error definitely indicate that the mean incubation period is a function of protein concentration.

To establish a definite mathematical relationship, it is necessary to determine the nature of the regression, that is, whether it is linear or whether it follows some other curve. That the relation is linear is shown by the close correspondence of the correlation coefficient $r=0.849$ and the correlation ratio $\eta=0.850$ (calculated from incubation-period variables); the difference, $\eta^2-r^2=0.002\pm0.003$, is less than 2 times its standard error and hence well within the limits of chance variation. In figure 4 the grouping of observed mean responses about the straight line (AB) drawn by the method of least squares further illustrates this linear relationship. Of major importance was the finding that the linear relation existed over the entire range of all protein quantities employed, that is, from $10^{-5.000}$ gm. (pI 5.0) to the smallest quantity, $10^{-9.515}$ gm. (pI 9.515) giving lesions.

The incubation-period relation may be applied in two general ways to the estimation of infectivity. The first of these consists in the use of the quantitative relation as a standard of reference for comparison with results obtained with an unknown material. This is permissible so long as

TABLE 3.—*Correlation of incubation period with papilloma protein—combined data of four experiments*

Incubation period	pI																Total	Means of arrays
	5.000	5.301	5.602	5.903	6.204	6.505	6.806	7.107	7.408	7.709	8.010	8.311	8.612	8.913	9.214	9.515		
Days																		
9–10	12																12	5.000
11–12	18	6	2														26	5.116
13–14	7	4	3			2											16	5.376
15–16	5	3	8	4	3	7											30	5.783
17–18		4	4	8	8	14	11	5	2								56	6.371
19–20			1	5	5	9	9	7	9	10	6	1					62	7.029
21–22				1	2	8	9	13	6	5	8	1	1				54	7.185
23–24		1				1	9	9	14	7	4	3					48	7.326
25–26							1	1	3	5	3	4	2		1		20	7.920
27–28								2	1	7	6	5		3			24	7.997
29–30						1		2	5		5	3	3	3			22	7.970
31–32											1			1	1	1	4	8.913
33–34															1	1	2	9.364
Total	42	18	18	18	18	42	39	39	40	34	33	17	6	7	3	2	376	
Means of arrays	11.7	14.6	15.4	17.8	18.2	18.6	20.5	21.9	23.0	23.1	24.3	25.8	26.8	28.9	30.2	32.5		

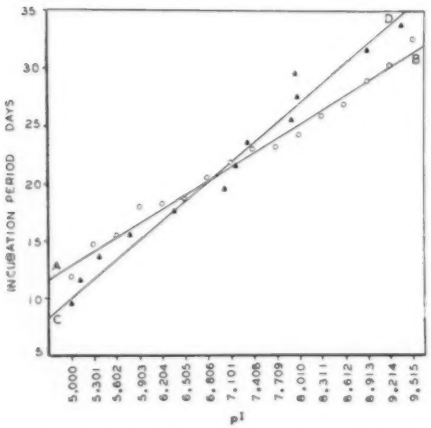

FIGURE 4.—Observed means of arrays and calculated regression lines from data of table 3. Line AB (calculated) and circles (observed means) show the regression of incubation period on protein concentration; line CD (calculated) and triangles (observed means) show the regression of protein concentration on incubation period.

the group sensitivity of the test-animal population remains uniform.

The regression line AB of figure 4 is a standard dosage-response curve described by the straight line equation,

$$y = a + bx, \tag{1}$$

where y represents the average latent period, in days, corresponding to any dose x, in terms of pI, and where a and b are constants for a given standard curve. The analysis of data when the response curve is linear has been discussed at length by Irwin (*22*). The constancy of the slope of the standard response curve as determined in the present work is evidenced by the relatively small standard error of the regression coefficient $b = 4.096 \pm 0.26$, a standard error of ± 6.4 percent in slope. This standard can be useful directly only with recognition of the variations inherent in such standard curves, caused for instance by random sampling, as indicated by the standard error of the regression coefficient, and by differences in populations of test animals. Considered as a standard for reference, the curve can be used either graphically or algebraically.

For the graphic application when serial dilutions of unknown or test materials are inoculated in the usual way, a response curve can be constructed by the method of least squares from the weighted mean incubation periods of the various dilutions. The line thus obtained may then be compared directly with the predetermined standard curve, in this instance that of AB in figure 4. The test line obtained will possess, within the limits of random sampling, the same slope as this line but may be displaced horizontally from it. The horizontal distance between the standard curve and that of the unknown represents the logarithm of the potency ratio. The same result may be obtained more readily by the use of a nomogram (fig. 5), representing a family of parallel lines corresponding to different standard dosages for constant dilutions. The test response curve may be located on the nomogram, and the position between standard oblique lines interpolated; or the mean incubation period

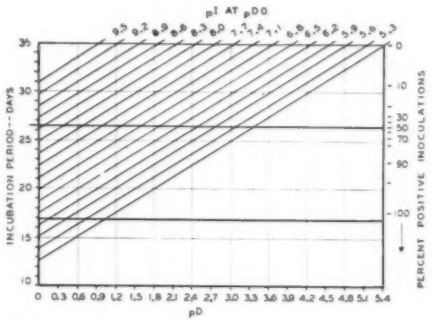

FIGURE 5.—Relation of incubation period to papilloma protein concentration and to the frequency of positive inoculations ($y = a + 4.096$ pI, where y = incubation period in days). The lower horizontal line represents the relations concerned with the complement-fixation unit of the protein.

of all dilutions giving lesions and the corresponding mean dilution may be plotted as coordinate points and interpolated in like manner.

Use may also be made of the equation for the standard response curve for algebraic comparison. In the present case this equation is

$$(2)\ \text{Incubation period} = -7.768 + 4.096\,pI.$$

The mean incubation period determined by observation on the results of inoculation of successive dilutions of the test material may be substituted in the equation above, and the pI of the corresponding mean dilution calculated.

For more accurate work the second general type of application may be used. Variations in susceptibility of test animals which shift the line horizontally to the left or right, as illustrated in a subsequent section, may be ruled out by simultaneous inoculation of both standard (prepared on the basis of protein determinations) and test materials in the same rabbits. In this case both the standard and test responses are determined, and the potency ratio between them established through substitution in the equation

$$(3)\ \text{Log}\left(\frac{\text{potency of test}}{\text{potency of standard}}\right) = pD_2 - pD_1 - \frac{y_2 - y_1}{b}$$

where pD_1 and pD_2 represent the respective mean dilutions of standard and unknown; y_1 and y_2 the corresponding observed mean incubation periods in days; and b the slope of a previously determined standard curve such as AB of figure 4. The pI of the standard inoculum being known, that of the test material is therefore readily calculable. The significance of the difference between responses may be determined by usual statistical procedures from the results of each assay or from previously determined standard deviations of standard response curves (cf. Irwin (*22*)).

Typical findings with this method and comparison of results with other procedures may be illustrated in a specific instance. The experiment consisted of 2 simultaneous assays of the same material, one on each side of the body, in a group of 12 rabbits. The response of the 12 animals with respect to positive inoculation sites is shown in table 2. On the basis of incubation periods of all lesions with the various dilutions employed in the 12 animals, an analysis was made comparing the response observed on one side of the body with that of the opposite side. The response curve for each side was calculated by the method of least squares giving appropriate weight to the mean incubation period for each dilution. These lines are shown in figure 6 and compared as described above. It will be observed that the 2 lines have essentially the same slope but are separated by a mean horizontal distance corresponding to the log ratio 0.030, or a difference of 7.2 percent in protein concentration as estimated from the 2 sides of the same animals (table 4).

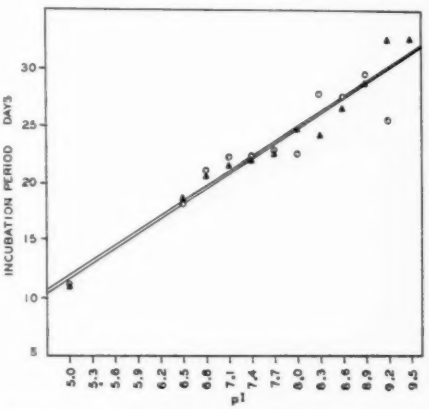

FIGURE 6.—Incubation-period response curves from right and left sides of rabbits (table 2) calculated by the method of least squares.

TABLE 4.—*Difference in apparent infectivity of identical inocula as measured by various procedures in opposite sides of 12 rabbits (table 2) and in subgroups of low and high response, A and B, respectively, of the same rabbits*

Method of comparison	Procedure					
	Left and right sides			Groups A and B		
	Incubation period	50-percent end point	Mean end titer	Incubation period	50-percent end point	Mean end titer
Log potency ratio	0.030	0.272	0.250	0.224	1.046	1.013
Potency ratio	1.072	1.870	1.778	1.675	11.117	10.292
Percent difference	7.2	87.0	77.8	67.5	1,011.7	929.2

From table 2 it is seen that the positive inoculation response of individual animals varies over an extremely wide range. If rabbits 23, 22, 21, and 16 are considered as a separate group (A) and compared with rabbits 15, 13, 14, 17, as a second group (B), extremes of response in the present instance are obtained. The incubation-period response curves for these two groups are compared in figure 7. Again the slopes of the two lines are almost identical, but the lines are separated by a greater horizontal distance than in the preceding analysis. Here the log ratio 0.224 corresponds to a difference of 67.5 percent in protein concentration as estimated in the two groups (table 4). Such variations in group susceptibility are probably extreme and unlikely to be frequent under conditions of random sampling.

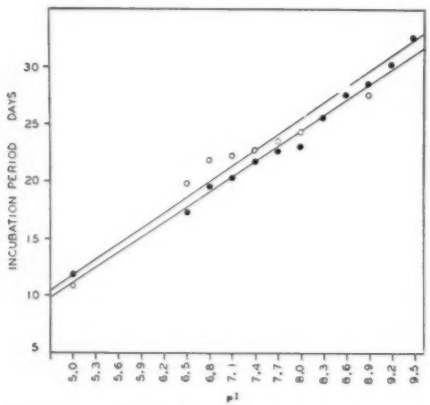

FIGURE 7.—Incubation-period response curves from groups A and B calculated by the method of least squares.

The results of this type of analysis may be compared with those concerned with end-point titer and with the 50-percent-point procedure suggested by Parker and Rivers (*19*) for vaccinia. In the same experiment with the 12 rabbits of table 2, the mean end-point titer for inoculation in the left side was found to be pI 8.286 while that for the right side was pI 8.536, a difference of 77.9 percent in infectivity with the same material in the 2 sides. In comparing the responses of the resistant group, A, with the more susceptible group, B, a large difference was observed. The mean end titer in group A was pI 7.850 and that of group B was pI 8.862, a difference of 900 percent in the response of the 2 groups to the same material.

Examination of the data was made also by means of the 50-percent-point procedure. The 50-percent-point inoculum for the left sides of the group of 12 rabbits was pI 8.215, and for the right side pI 8.487, a difference of 87 percent. The 50-percent response of animals of group A was at pI 7.829, and that for the susceptible group at pI 8.875, a difference of more than 1,000 percent. These results with the different methods are summarized in table 4.

The close correlation of the papilloma protein with incubation period demonstrated in the foregoing experiments has been corroborated in a large mass of data accumulated in numerous experiments described in subsequent sections of this paper. The relation has held consistently in results obtained with protein from numerous different batches of warts. These observations provide striking evidence both of

the uniformity of the infectious properties of the papilloma protein and of the high degree of accuracy to be expected through use of the incubation-period relation in the estimation of infectivity.

Correlation of Incubation-Period Relations With the 50-Percent-Point Infectious Unit of Papilloma Protein

Considerable stress has been placed on the comparison of results obtained with the incubation-period relation with those of end-point and 50-percent-point titration of the papilloma protein. In the experiments cited, it was evident that the incubation-period procedure furnished a much more accurate estimate of infectivity than did either of the other two methods and this has been repeatedly corroborated. One point should be noted here, however. On an absolute basis, it cannot be said that the incubation-period method is more accurate than the end-point titer or 50-percent-point analysis. The reproducibility of results with either procedure obviously depends on the conditions of the experiment. For a given number of rabbits used in an experiment, far greater accuracy is to be expected from the incubation-period analysis. On the other hand, an equal degree of accuracy might be obtained with the end-point titer or 50-percent-point estimation in a larger group of animals. However, in the instance of the papilloma virus unless prohibitive numbers of animals are used, statistical analysis of these end-titer results shows that the observed mean values are not sufficiently reliable for quantitative work. Utilization of the 50-percent point for reference provides larger numbers of applicable observations than simple end-point determination and thus greater reliability in interpretation, but here, too, the number of animals necessary is impractical for routine large-scale work. Quantitatively, then, the differences in the results with the different methods are entirely relative, but for routine experiments employing a practical number of animals, the incubation-period relation furnishes results of greater accuracy than do either the 50-percent-point procedure or end-titer determination.

It is thus evident that the incubation-period analysis furnishes advantages over either the end-titer or the 50-percent-point procedure. A serious impediment to the use of the incubation-period method alone, however, exists in the lack of a convenient unit or point of reference for the description of infectivity. It could be said that a given preparation was a 13-day (incubation period) virus, or perhaps the standard regression line could be extended to intercept the incubation-period level corresponding to the period of the smallest amount of virus ever observed to give a lesion. Such consideration would be cumbersome and would furnish no means for correlating the infectious properties of the papilloma virus with those of other filtrable agents.

On the other hand, the 50-percent point has a common significance with respect to all viruses since this point is an estimate of the median, or an average quantity of virus required to just produce a lesion in the test-animal population under consideration. If, by the use of a large number of test animals, the incubation period observed for the dose of virus which gives 50-percent positive inoculation sites can be shown to be reproducible, within the limits of chance variation, then a standard relation between the 50-percent-point dose and its incubation period could be established empirically. Insofar as test-animal sensitivity is stable, therefore, the specific latent period of the standard 50-percent-point dose may be used along with the slope of the standard response curve for estimating the 50-percent dose of test mate-

rials on the basis of incubation period data alone. In the work described here, experiments were made (*23*) to determine in a suitable number of animals the amount of protein giving the 50-percent point and to correlate this point, if sufficiently stable, with the standard-incubation-period relations.

Three groups of data have been analyzed. Two of these were the results of single experiments, and the third was obtained by combining observations in several experiments.

In experiment 1 twofold dilutions of papilloma protein of pI 5.0 through 9.5 were prepared from a single specimen of purified material. Inoculations of pI 5.0 and of pI 6.5 through 9.5 were made in duplicate in each rabbit. The range pI 5.3–6.2, inclusive, was omitted because of lack of space on the rabbits, but previous experience has shown this pI region to yield lesions consistently at every point inoculated.

Experiment 2 was made in the same manner as the preceding, except that a different batch of protein was used. One of the twelve animals inoculated died before completion of the experiment.

In experiment 3 are grouped the results of several series of inoculations of the lower pI values (higher concentrations of protein). This compilation involved studies on 5 different batches of purified protein and included observations on 22 rabbits.

In table 5 are shown the data of these experiments combined. Included are observations on 7 different batches of protein in a total of 45 test rabbits in which from 20–72 inoculations were employed for each pI. It is seen that the pI_{50} for the combined data was 8.355. Analyses were made also for each of the 3 groups of data separately. The respective determined 50-percent points were pI 8.353, 8.277, and 8.485. Within the limits of this small series of experiments, the extreme difference was that between pI 8.277 and 8.485, a difference of 0.208, or approximately two-thirds of a dilution interval.

The criterion employed in the present experiments for the estimation of papilloma protein was turbidity measurement, and thus the relation between the observed 50-percent point and protein was established on this basis. The incubation-period relations previously described were likewise based on protein estimations made

TABLE 5.—*Calculation of 50-percent point from combined data of three experiments* [1]

pI	Rabbits	Total inoculations	Inoculations		Accumulation		Percent positive
			Positive	Negative	Positive	Negative	
	Number	*Number*	*Number*	*Number*			
5.000	22	25	25	0	550	0	100.00
5.301	19	22	22	0	525	0	100.00
5.602	17	20	20	0	503	0	100.00
5.903	19	22	22	0	483	0	100.00
6.204	17	20	20	0	461	0	100.00
6.505	37	63	63	0	441	0	100.00
6.806	45	71	68	3	378	3	99.21
7.107	42	68	63	5	310	8	97.48
7.408	40	66	62	4	247	12	95.37
7.709	42	68	58	10	185	22	89.37
8.010	42	68	58	10	127	32	79.87
8.311	33	59	32	27	69	59	53.91
8.612	30	56	16	40	37	99	27.20
8.913	26	52	10	42	21	141	12.96
9.214	29	55	7	48	11	189	5.50
9.515	26	52	4	48	4	237	1.66

[1] 50-percent point $= 8.311 + \frac{53.91 - 50.00}{53.91 - 27.20}(0.301) = \text{pI } 8.355.$

by the same method. It follows that a quantitative relation exists between incubation period and the 50-percent point.

The results show that the 50-percent point, as determined by analysis of the frequency distribution of positive and negative lesions, is a point of high probable stability under the specified standard conditions which include utilization of sufficiently large numbers of animals for adequate analyses. As such, it can be considered as a standard and related directly to the standard incubation-period response curve. This is illustrated in figure 8. The 50-percent point, pI 8.355, found in the combined data of table 5, is located on the standard response curve at the level of the standard incubation-period response 26.45 days, corresponding to the inoculum pI 8.355. On this curve, which was determined by experiments with known quantities of protein, the 50-percent point is located as a single point. On the standard nomogram (fig. 5) representing graphically the series of standard response curves of materials of various initial strength, that is, zero dilution, the 50-percent-point relation can be shown in a line level corresponding to 26.45 days.

From this standard relation, the pD_{50}, or dilution of a test material to be expected to give an equal number of positive and negative inoculations, can be calculated from incubation-period data obtained in a small group of animals without actually using the dilutions falling within the range necessary for direct determination. This can be accomplished algebraically or graphically. In an experiment with material of unknown potency inoculated in serial twofold dilutions, the mean dilution, pD_m, corresponding to the mean incubation period y_m, is determined. By substitution in the equation

$$(4) \qquad pD_{50}=pD_m+\frac{26.45-y_m}{4.096}$$

the pD_{50} is derived. The standard nomogram may also be used. The response curve of the test material may be located on the nomogram of figure 5, and the dilution corresponding to the point where the test response curve intersects the standard 50-percent-point response level is located graphically and the pD_{50} learned by interpolation. A better estimate would be obtained by plotting the mean incubation period and the corresponding mean dilution as coordinate points on the nomogram and drawing a line through the point parallel to the standard response curves. The pD at the intersection of this line with the standard 50-percent-point level is the pD_{50}.

Estimation of Median Individual Effective Virus Dose

If the 50-percent point is accepted as a stable point, which it has appeared to be under present conditions, it can be used for the numerical expression of potency or infectivity of given tested papilloma-protein preparations. The 50-percent point, implying a definite relation of quantity to response, can be considered a standard unit for describing infectivity. In the present instance, this infectious unit corresponds to pI 8.355. It should be em-

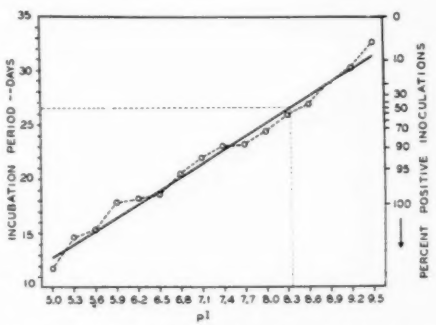

FIGURE 8.—Relation of positive inoculations to the standard incubation-period response curve AB, fig. 4).

phasized that the symbol pI implies not only a given quantity of protein but indicates that this quantity is included within a definite standard volume. A 50-percent-point infectious unit of papilloma protein is then $10^{-8.355}$ gm. of protein in 0.1 cc. of inoculum. In view of the relations established between this unit and the incubation period, the potency of materials investigated by means of the latter relation can be expressed in terms of infectious units. The number of infectious units present in the mean dilution, pD_m, corresponding to mean incubation period, y_m, can be determined by substitution in the equation,

(5) Log infectious units (at pD_m) $= pD_{50} - pD_m$, or

(6) Log infectious units (at pD_m) $= 8.355 - \frac{y_m + 7.768}{4.096}$

The 50-percent-point values which can be thus calculated will be subject to the same degree of variation as that inherent in the determination of incubation-period response curves in different groups of test animals. The limits of variation, then, are the same as those for variations in observed incubation-period response curves about the standard curve for a given population of test animals.

The size of the papilloma protein molecule or elemental unit has been estimated from the results of centrifugation and of filtration. By the former method, Beard and Wyckoff (*10*) considered its diameter to be about 40 millimicrons while Schlesinger and Andrewes (*21*) gave the values 32–50 mμ. By means of ultrafiltration the latter authors arrived at the values 23–35 mμ. If the outside limits of the diameter estimated by Schlesinger and Andrewes, 32–50 mμ, obtained by assuming the specific gravity of the virus particle to be 1.3 or 1.4 are averaged, the value found is 41 mμ. The value, 40 mμ, has been chosen for the present purpose.

Assuming the specific gravity of the papilloma protein molecule to be 1.4, the weight of the molecule of 40 mμ diameter would be $10^{-16.329}$ gm. The 50-percent-point infectious unit, pI 8.355, therefore, would consist of about 94,000,000 molecules of papilloma protein per 0.1 cc. inoculum.

HOST INFLUENCE IN CHARACTERIZATION OF RESPONSE TO PAPILLOMA PROTEIN AND TO VACCINIA AND INFECTIOUS MYXOMA VIRUSES

In the preceding discussions infectivity of the papilloma virus has been considered essentially from the point of view of the virus. It should be remembered, however, that neither the qualitative nor the quantitative manifestations of infectivity are referable to properties of the virus alone. In the infectious process, both virus and host participate in mutual interactions to characterize the final result. Indeed, biologically, virus effect is recognizable and measurable for the most part in terms of host response rather than on the basis of criteria applicable to the virus alone. That host influence is of paramount significance in the characterization of response to viruses has long been recognized. From this point of view it is clear that the concept of degree of infectivity should include proper consideration of both host and virus factors. In the instance of the papilloma virus, under the standardized conditions of the experiments the 50-percent-point infectious unit is about 94,000,000 molecules. From this result one could consider the papilloma virus to be of low infectivity. On the other hand this result was found under a given set of conditions and constitutes a measure

of the mean interaction of the virus and a particular host, the domestic rabbit. The experiments have shown that the purified papilloma virus is a material of uniform behavior, that is to say, a uniform proportion of the individual molecules in the various preparations must possess infectious properties. To bring about a given effect in the domestic rabbit a large number of molecules of uniform behavior acting together are necessary. On this basis, it is evident that the quantitative aspects of the 50-percent-point infectious unit are actually as much a measure of the susceptibility or resistance of the domestic rabbit as of the infectious properties of the virus unit itself.

As has been shown, individual domestic rabbits vary greatly in their response to inoculation with the papilloma virus, though the mean response of various groups of animals is highly uniform. These differences in individual virus-host interactions considered as a whole constitute a pattern of response. In the experiments already described, the mean effect of the virus, or conversely the mean response of the host, has been determined through standardization of host conditions through the use of large numbers of animals. In this section of this paper are considered the participation or the influence of host factors or differences in the characterization of the response pattern to standardized preparations of purified virus. Much of the interest in this phase of the problem has arisen through findings by Parker (*25*) in studies on vaccinia virus. Interpretations of the results with the papilloma virus are greatly dependent on analysis of Parker's results with vaccinia, and for this reason the results obtained with both vaccinia and the papilloma viruses are described together. An analysis has been made also with data on the virus of infectious myxoma recently reported by Parker (*26*).

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Character of Dose-Frequency-Response Curves to Vaccina and Myxoma Viruses in Relation to Response to Papilloma Virus

In investigating quantitatively the infectivity of purified preparations of vaccinial elementary bodies, Parker and Rivers (*19*) examined the frequency distribution of positive and negative inoculations with graded numbers of the bodies. Plotting the percentage occurrence of lesions appearing at those sites receiving a given inoculum against the number of elementary bodies present in the inoculum, a response pattern in the form of an S-shaped distribution curve was obtained. In a subsequent study of this curve Parker (*25*) noted its resemblance to Poisson curves relating to the distribution of small particles in suspension. The curve with vaccinia virus was found to be more nearly like the Poisson prediction of the distribution of one or more particles than any other of the series. Wholly on the basis of this resemblance, Parker advanced the concept that a single vaccinial elementary body constituted an infectious unit of the virus and that the character of the S-shaped curve was thus due entirely to the presence or absence of virus particles in the inocula or, in other words, to factors concerned with the virus alone.

In the work concerned with the determination of the 50-percent-point infectious unit of papilloma virus, the frequency distribution of positive and negative inculations was studied. When the results were expressed graphically, S-shaped curves were obtained, and analyses of the distribution by the procedure used by Parker revealed a very close resemblance between the findings with the papilloma virus and those with vaccinia virus. This is illustrated in the results of a typical experiment.

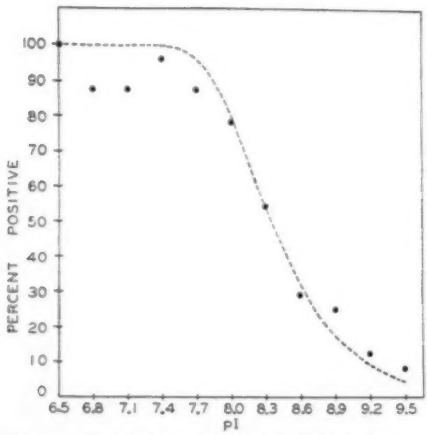


FIGURE 9.—Relation of observed frequency points to calculated theoretical curve expected, if a single virus particle causes infection. Data of table 6.

For this the results obtained with the 12 rabbits of table 2 were used. These animals were inoculated with twofold dilutions of protein of pI 5.0 and 6.5 through 9.5. Inoculations of each dilution were made in duplicate in each animal on both sides of the midventral line. The range pI 5.3–6.2, inclusive, was omitted because of lack of space on the animals, but previous experience had shown this region to yield lesions consistently at every site inoculated.

The observed frequency distribution of positive and negative inoculation sites is given in table 6 and shown graphically in the points of figure 9. In table 6 are given also the theoretical frequencies calculated from the Poisson law. If 50 percent of the unit volumes taken from a given preparation (here the dilution corresponding to the 50-percent point) contains no particles at all, it can be determined from the Poisson law that the mean number of particles per unit volume of this preparation is 0.69. From this relation the theoretical frequencies for the various other dilutions can be found. These theoretical frequencies, calculated on the assumption that 1 molecule of papilloma protein causes infection, are compared with the observed frequencies in table 6. The theoretical curve corresponding to the calculated frequencies is shown in the broken line of figure 9.

The observed points fall fairly close to the theoretical curve. If one considers only the positive frequencies, as did Parker, and calculates the χ^2 for goodness of fit, it is seen in this experiment that the proba-

TABLE 6.—*Data of the experiment of table 2. Comparison of observed with theoretical frequencies* $(d=x_0-x_T=y_0-y_T)$

pI	Inoculations	Inoculations					d	$\frac{d^2(x_0+y_0)}{x_T y_T}$
		Positive			Negative			
		Observed (x_0)	Theoretical (x_T)	$\frac{(x_0-x_T)^2}{x_T}$	Observed (y_0)	Theoretical (y_T)		
	Number							
6.5	24	24	24.00	0.00	0	0.00		
6.8	24	21	24.00	.37	3	.00		
7.1	24	21	24.00	.37	3	.00		
7.4	24	23	23.97	.04	1	.03	9.04	5.36
7.7	24	21	22.97	.17	3	1.03		
8.0	24	19	19.02	.00	5	4.98		
8.3	24	13	13.08	.00	11	10.92		
8.6	24	6	7.82	.42	18	16.18	1.82	.63
8.9	24	6	4.29	.68	18	19.71	1.71	.83
9.2	24	3	2.25	.25	21	21.75	.75	.27
9.5	24	2	1.15	.63	22	22.85	.85	.66
		$\chi^2=2.93$ P=0.98				$\chi^2=7.75$ P=0.10		

bility of a worse fit occurring by chance than that actually found is quite high.

Similar findings in other experiments with the papilloma protein parallel those reported for vaccinia virus. With both viruses conformity of the observed with the theoretical frequencies appears probable, and it could be concluded with equal assurance for both that the character of the distribution is determined by the chance presence or absence of virus particles in the inocula.

Despite the evident conformity of the observed results with the theoretical frequencies, this concept, nevertheless, seems unsatisfactory. In the exclusion of all possible host participation in determining the character of distribution, the hypothesis is contrary to general experience. Further, it is at variance with direct observations on the infectivity of the vaccinia and papilloma viruses. For the former, infectious units of about 1,000, 300, and 4 elementary bodies, respectively, have been observed in studies (*27–29*) with different strains of virus. On the basis of virus particle number the magnitude of the discrepancy between theory and observation in the instance of the papilloma virus is of the order of 10^4–10^6 times that for vaccinia. While it might be possible, though somewhat remotely so, to explain the findings with vaccinia on the basis of inhomogeneity of preparation, the uniformity of infectivity results renders such an explanation entirely untenable in the instance of the papilloma virus.

In seeking an approach to an explanation of the differences between theory and experimental findings (*30*), the results with both vaccinia and the papilloma viruses were analyzed further. Parker's interpretation of his results rested on the similarity of the theoretical to the observed distribution when the conformities of the two were tested on the basis of percentage of positive inoculations alone. On the other hand, when the χ^2 of both positive and negative inoculations were combined as suggested by Haldane (*31*), the values of P in Parker's four experiments (*25*) were 0.73, 0.71, 0.006, and <0.001, respectively. Thus in two of his experiments the deviation of observed from theoretical results was beyond the limits of chance variation since in both P was less than 0.01. In the findings with a similar examination of the papilloma virus, a consistent deviation was observed in individual experiments which assumed significant proportions only when several experiments were combined. This is illustrated in table 7 and figure 10. It should be noted that such a combination of the data from several experiments is permissible since the pI of papilloma virus of the respective inocula was the same in all experiments.

An obvious solution to the problem existed in the possibility that the distribution might not be a Poisson curve at all but might be owing to factors other than those related to the virus alone. It seemed likely that any hypothesis suitable for the prediction of the course of infection, such as the frequency distribution of positive and negative inoculations, should include

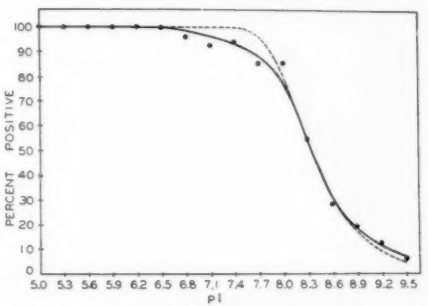


FIGURE 10.—Observed frequency (represented by points through which the solid line was drawn by inspection) and theoretical curve (broken line) expected if one virus particle causes infection. Data of table 7.

TABLE 7.—*Combined data of three experiments* ($d = x_0 - x_T = y_0 - y_T$)

pl	Inoculations	Inoculations					d	$\frac{d^2(x_0+y_0)}{xt;t}$
		Positive			Negative			
		Observed (x_0)	Theoretical (x_T)	$\frac{(x_0-x)_T^2}{(x_T)}$	Observed (y_0)	Theoretical (y_T)		
	Number							
5.0	25	25	25.00	.....	0	0.00		
5.3	22	22	22.00	.....	0	.00		
5.6	20	20	20.00	.....	0	.00		
5.9	22	22	22.00	.....	0	.00		
6.2	20	20	20.00	.....	0	.00		
6.5	63	63	63.00	.....	0	.00		
6.8	71	68	71.00	0.00	3	.00	14.28	12.14
7.1	68	63	67.98	.36	5	.02		
7.4	66	62	65.86	.23	4	.14		
7.7	68	58	64.91	.73	10	3.09		
8.0	68	58	53.53	.37	10	14.47		
8.3	59	32	32.75	.02	27	26.25	.75	.04
8.6	56	16	17.94	.21	40	38.06	1.94	.31
8.9	52	10	9.13	.08	42	42.87	.87	.10
9.2	55	7	5.07	.73	48	49.93	1.93	.81
9.5	52	4	2.44	1.00	48	49.56	1.56	1.05
		$\chi^2=3.73$ P=0.92				$\chi^2=14.75$ P=0.01		

consideration of both host and virus factors. Such has been found to be the case in the study of biological response to materials other than viruses, chiefly drugs, in which the results have been found to follow certain well-defined laws.

Normal Distribution of Variations in Host Susceptibility in Response to Vaccinia and Myxoma Viruses

Burn (*32*) has pointed out that with biological responses which are measurable, the relation between the logarithm of the dose and the mean effect in a group of animals is usually linear. Such a linear relation between incubation period and logarithm of dose has already been demonstrated for the papilloma protein. When the response is all or none, such as death or survival, presence or absence of lesions, etc., there is a range of doses which have no effect on any animal, a range in which each dose is effective in every animal, and an intermediate range in which the percentage of positive responses increases with the dose. When the logarithm of dose is plotted as abscissa against percentage responses as ordinate, an S-shaped response curve is obtained, the slope of which is constant for a given material and a given population of test animals. The character of S-shaped dose-mortality curves obtained with certain drugs has been attributed to variations in animal response. Behrens (*33*) studying the effect of strophanthin in frogs found that variations in the lethal dose followed a frequency distribution resembling a normal curve. By expressing the class frequencies as percent and accumulating the latter from the lower toward the higher doses, an S-shaped "integrated percentage frequency" curve was obtained (*32*) which was identical with a dose-percent response curve obtained by Trevan (*34*). Commenting upon this finding, Burn states, "It is evident from the agreement that Trevan's dose-mortality curve is to be explained as an integrated frequency curve." The possibility exists that the character of the dose-response curves for both vaccinia and the papilloma protein may be determined, not by the chance presence or absence of virus particles in inocula, but rather by variations in the response of test animals. This possibility has been examined on the

assumption that the dose-response curves observed with these viruses are integrated frequency curves.

When variations in animal response follow a normal frequency distribution, a straight line relation exists between the logarithm of dose and "normal equivalent deviation" (*35*). The latter is obtained by converting the observed percentage response into the deviation on a normal frequency curve to which the percentage is equivalent. An example of the conversion of Parker's data is illustrated in table 8. These data were taken from Parker (*25, table 1*). From the number of inoculations and the number of these which gave positive results, the percentage positive was calculated. From published tables (*36*) the values of the normal equivalent deviation corresponding to the respective percentages were found. These values were plotted against the logarithm of virus dilution.

TABLE 8.—*Expression of percentage positive inoculations as normal equivalent deviation* [1]

Logarithm of virus dilution	Inoculations			Normal equivalent deviation (S. D. units)
	Total	Positive		
	Number	*Number*	*Percent*	
6.7	69	67	97.10	1.8981
7.0	69	58	84.06	.9970
7.3	72	49	68.05	.4691
7.7	71	20	28.17	−.5778
8.0	72	14	19.44	−.8619
8.3	75	6	8.00	−1.4051
8.7	76	1	1.31	−2.2418

[1] Data of columns 1 to 3 from Parker (*25, table 1*).

The results obtained for the data of Parker's experiments 1 and 4, given in his tables 1 and 4, are shown in figures 11, *A*, and 12, *A*, respectively. In each figure the straight line was drawn through the observed points by the method of least squares. By inspection it is apparent that the points fall close to the straight lines. For each group of data, tests were made for the goodness of fit of the observed frequencies to those derived from the

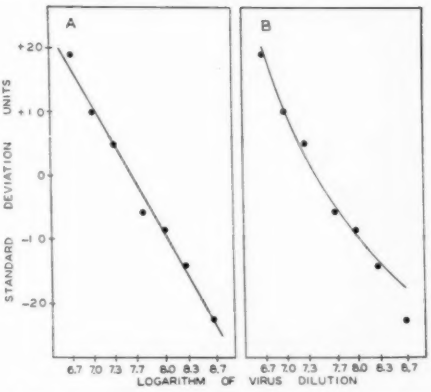


FIGURE 11.—Points represent observed percentage response to vaccinia virus in Parker's table 1 (*25, p. 730*) expressed as normal equivalent deviation. Deviation is shown in standard deviation units. *A*, Comparison of observed points with straight line drawn by the method of least squares; *B*, Comparison of observed points with curve determined from theoretical values given by Parker on assumption that a single virus particle infects.

expected normal equivalent deviation represented by this straight line. The expected frequencies were derived by a reverse of the procedure illustrated in

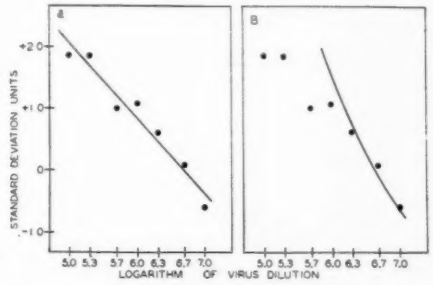


FIGURE 12.—Points represent observed percentage response to vaccinia virus in Parker's table 4 (*25, p. 734*) expressed as normal equivalent deviation. Deviation is shown in standard deviation units. *A*, Comparison of observed points with the straight line drawn by the method of least squares; *B*, Comparison of observed points with curve determined from theoretical values given by Parker on assumption that a single virus particle infects.

TABLE 9.—*Summary of probabilities found with Parker's experiments 1–4 on vaccinia virus*

[A. Comparison of observed frequencies with those expected if one virus particle causes infection. B. Comparison of observed frequencies with those expected if the relation between logarithm of dilution and normal equivalent deviation is linear]

Parker's experiment No.	P, for goodness of fit			
	A		B	
	$\chi^2=S\frac{(f_0-f_T)^2}{f_T}$	$\chi^2=S\frac{d^2(f_0+y_0)}{f_T\ y_T}$	$\chi^2=S\frac{(f_0-f_T)^2}{f_T}$	$\chi^2=S\frac{d^2(f_0+y_0)}{f_T\ y_T}$
1	0.90	0.73	0.90	0.67
2	.93	.71	.96	.94
3	.12	.006	.41	.19
4	.96	<.001	.98	.77

table 8. The results of the tests for the two experiments are summarized in table 9 (B). In table 9 (B) are shown also the results with Parker's experiments 2 and 3 (*25, pp. 731–734*). In all instances the observed frequencies correspond closely to those expected.

The results of these calculations may be compared with those derived in a similar way on the hypothesis that the character is due to the chance presence or absence of virus particles in the various inocula. The theoretical frequencies calculated by Parker on the assumption that one virus particle causes infection can be expressed in terms of percent and converted to normal equivalent deviation. When this is done, a curved line is obtained. If this theoretical curve is then fitted to the observed points shown in figures 11, *A*, and 12, *A*, at the point of zero deviation, which corresponds to the 50-percent point, the results found are those shown in figures 11, *B*, and 12, *B*. In figure 11 there is little to choose between the curved line of figure 11, *B*, and the straight line of figure 11, *A*. On the other hand, the straight line of figure 12, *A*, described the results much better than the curve of figure 12, *B*. Tests for the goodness of fit of the observed to the theoretical frequencies calculated from the Poisson law for each of these two experiments, as well as those of Parker's experiments 2 and 3, are compared in table 9 (A) with expected frequencies determined from the straight lines, table 9 (B). In all instances there was agreement of observed data with those expected if the relation is linear whereas in two of the experiments, that of figure 12, *B*, and Parker's experiment 3, the fit of the data to the theoretical Poisson curve was improbable.

It is of interest to examine the data described by Parker (*26*) in his study on the frequency distribution of positive and negative inoculations with the virus of infectious myxomatosis. As in the instance of the results with vaccinia virus, he found a close resemblance of the dose-response curve to the theoretical Poisson curve based on the assumption that the frequency distribution was due to the presence or absence of virus particles. In these experiments, however, Parker noted a systematic deviation of the observed points from the theoretical curve as was seen with the papilloma virus. The data obtained by Parker (*26*) with the virus of infectious myxomatosis in his experiment 1, table 1, have been analyzed as for vaccinia on the assumption that the character of the response was due to normal variations in host reactions. Figure 13, *A*, shows the observed points which fall close to the straight line drawn by the method of least squares. In figure 13, *B*, the observed points are compared with the theoretical

Poisson curve. It is thus clear that the characters of the frequency distributions are better explained as due to normal variations in host response than to the presence or absence of virus particles in the inocula.

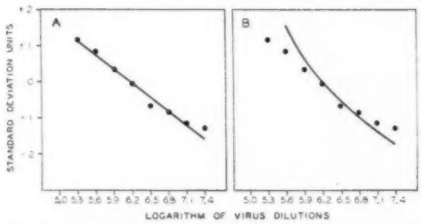


FIGURE 13.—Points represent observed percentage response to myxoma virus in Parker's table 1 (*26*) expressed as normal equivalent deviation. Deviation is shown in standard deviation units. *A*, Comparison of observed points with straight line drawn by the method of least squares; *B*, Comparison of observed points with curve determined from theoretical values given by Parker on assumption that a single virus particle infects.

RESISTANCE ACQUIRED IN LONG INCUBATION PERIOD AS A FACTOR COMPLICATING CHARACTER OF HOST RESPONSE TO PAPILLOMA VIRUS

Similar analyses were made for the observations with the papilloma protein. The findings are illustrated by the results with the data of tables 6 and 7, the latter of which represents the combined findings in three separate experiments. The observed frequencies of tables 6 and 7 were converted into normal equivalent deviation in the manner described for vaccinia.

The results are shown graphically in figures 14, *A*, and 15, *A*, respectively.

In neither of the figures do the points conform to a straight line distribution and a line drawn through the points must be curved. The variations evidently do not follow a normal distribution, and the distribution must be skewed. In figures 14, *B*, and 15, *B*, the theoretical Poisson curve has been drawn as for vaccinia, and the observed points likewise do not follow this curve. It is plain either that the fundamental nature of the response to the papilloma protein differs from that to

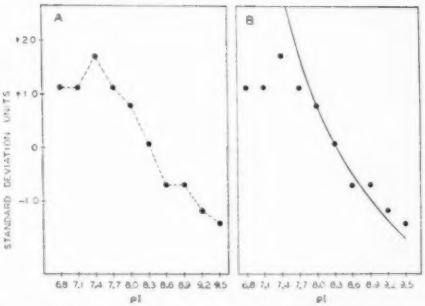


FIGURE 14.—Observed percentage response to papilloma protein, calculated from data of table 6 and expressed as normal equivalent deviation. Deviation is shown in standard deviation units. *A*, Observed points; *B*, Comparison of observed points with curve determined from theoretical percentages expected on assumption that a single papilloma protein molecule causes infection.

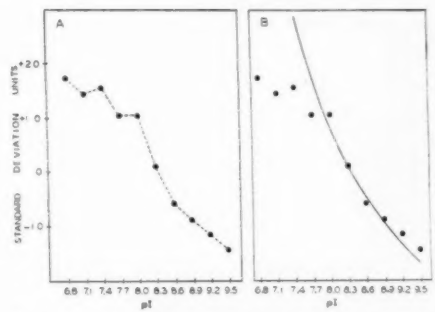


FIGURE 15.—Observed percentage response to papilloma protein calculated from data of table 7 and expressed as normal equivalent deviation. Deviation is shown in standard deviation units. *A*, Observed points; *B*, Comparison of observed points with curve determined from theoretical percentages expected on assumption that a single papilloma protein molecule causes infection.

vaccinia or that the difference in the results with the two viruses is due to factors absent or failing to exert an influence in the response to vaccinia. Further analyses of the above data provide evidence of the presence and the nature of such factors affecting response to the papilloma protein.

Domestic rabbits vary greatly in individual resistance to infection with the papilloma protein and to the development of warts, as illustrated in table 2. The sum total of this resistance may be a combination of two sorts, namely, (1) natural and (2) acquired resistance. Individual differences in natural resistance, that is, that present at the time of inoculation, are evident in deviations about the mean incubation period corresponding to a given inoculum observed when warts first begin to appear. Acquired resistance may act also in determining the final response to the virus. Within the incubation period 6–35 days there develops a state of immunity or increased resistance to infection on further inoculation with the virus. There may develop also a resistance directed toward the growth, and in some animals there is actual complete retrogression of warts which had appeared early. Rabbits chosen at random from mixed populations vary also in susceptibility or resistance to vaccinia virus, but the course of infection with this virus differs greatly from that with the papilloma virus. The incubation period of vaccinia is approximately one-fourth that of the papilloma protein. Lesions resulting from inocula containing much virus appear early, while those arising from small quantities of virus in the same animal appear later, even after the healing process of the early lesions is under way. This behavior differs from that observed with the papilloma protein for in any one animal once retrogression begins, no more warts appear. It has seemed possible that the character of the response curve of figure 15, *A*, may be the result of the change in the level of resistance known to occur during the course of the incubation period. To obtain information concerning the possible relation of this change to the character of the response, analyses have been made of the data observed at various intervals during the incubation period.

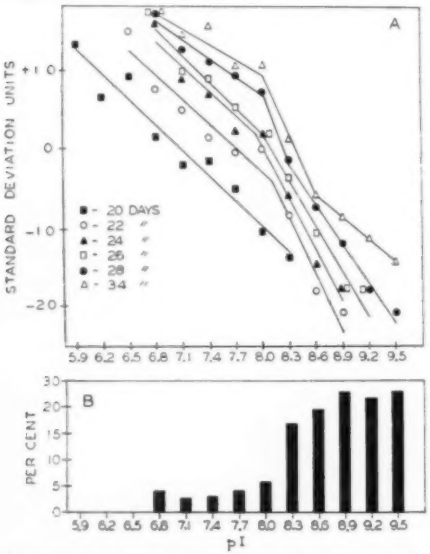


FIGURE 16.—*A*, Trends in response to the papilloma protein at various intervals after inoculation with the protein. (Combined data of experiments of table 7 employed in this analysis). *B*, Percentage of total number of inoculations not having a chance to become positive because of onset of retrogression.

For the following analyses the combined data given in table 7 have been used. From the total number of inoculations and the number of inoculations positive on various days during the period of incubation, the percentage positive corresponding to these days was found. The percentages were converted to normal equivalent deviation and the points plotted as described above. The resulting series of curves describing the character of response

to the various inocula observed after 20, 22, 24, 26, 28, and 34 days, are shown in figure 16, *A*.

The points representing the frequencies observed on the twentieth day are clearly distributed about a straight line. Examination of the data prior to this day showed that, though variations were more marked owing to the small number of observations available, the relation before the twentieth day was likewise linear. The successive changes in the character of the curves leading to the complicated response shown in the final curve of the thirty-fourth day were continuous and orderly. On the twenty-second day, two new points appeared, but these did not fall on the line determined by the points appearing earlier. A break thus occurred, and this increased with time. At 24 and 26 days the positive frequencies were distributed so that the points could be considered to lie about two straight lines with different slopes. At 28 days there was an indication of a second break, which occurred at pI 8.6. At this period it seems that the points were distributed about three straight lines and this possibility was emphasized in the definite breaks seen in the curve of 34 days.

In the present work no specific experiments were made to relate the time of development or the degree of induced immunity to the character of the response. Notes were made, however, in instances where retrogression occurred in individual animals. If the onset of actual retrogression in some of the rabbits is taken as an index of time of change owing to development of acquired resistance in the group, a comparison of this change in resistance with the character of the curve can be made. The relation is shown in figure 16, *B*. The respective columns beneath the curves were derived in the following manner: It has been shown that successive dilutions of the protein induce growths after correspondingly long incubation periods. Since warts do not appear in those sites inoculated with the smaller amounts of protein after retrogression sets in, the last dilution producing warts in the individual animals showing retrogression can, therefore, be taken as a dividing line between the dilutions which had opportunity to produce warts and those which did not have this opportunity because of the host factors indicated by retrogression. The heights of the columns in figure 16, *B*, indicate the percentage of the total number of inoculations with each pI which did not have the opportunity to cause lesions. The regions of curvature or breaks in response correspond closely to the dilution range where the group response is complicated by individual test-animal resistance. It is not to be considered that only those animals showing retrogression contribute to the change in group reaction; for all domestic rabbits infected with the virus develop increased resistance to further infection with the virus and all of the animals will contribute in varying degree.

On the basis of these findings it is justifiable to assume that the character of the final curve may be determined by variations in host response. Viewed in this light, a possible interpretation of the curve is at once suggested. For purposes of discussion, the curve is divided into three components, namely, (1) that between pI 6.8 and 8.0; (2) that between pI 8.0 and 8.6; and (3) that between pI 8.6 and 9.5. It will be seen that component 1 is linear in the thirty-fourth day curve and that this relation was present on the twentieth day and maintained throughout the course of the incubation period, showing only a change in slope. It thus seems evident that the character of this component was due to host variations present early, namely, to variations primarily in natural resist-

ance. Skipping component 2 for the moment, it appears that component 3 represents the response of those animals in which warts continued to appear throughout the incubation period and thus may be interpreted as a linear relation owing to the normal distribution of total resistance in these animals. Component 2, then, would represent the response manifested during the rapid change in the level of resistance in the group as a whole. There is evidence from the series of curves that component 2 is probably linear over most of its course. It will be noted that this component is derived from the linear relation established first on the twenty-second day and maintained through the subsequent orderly addition and loss of points. It is not unreasonable to interpret component 2 as a straight line, for this would mean simply the existence of normal variations in the development of acquired resistance in the individual animals.

It should be noted here that in some instances very slight deviations from the straight-line dose-response curve similar to the large differences with the papilloma virus are seen with vaccinia. This is well shown in the curve of figure 6 of a previous report (*30*).

COMPLEMENT-FIXING CAPACITY OF THE PAPILLOMA PROTEIN

With the development of the incubation-period procedure two criteria were at hand for accurate practical assay of the purified papilloma virus, namely, (1) mass measurement by physical and chemical analysis and (2) biological measurement in the estimation of infectivity. To add to the knowledge of the behavior of the papilloma protein and to furnish quantitative data useful in the recognition and assay of the virus, another relation has been studied. The purified papilloma protein fixes complement in the presence of specific immune serum as does the virus of crude infectious extracts of cottontail-rabbit warts (*37*). In routine work with the papilloma protein over a period of several years, the complement-fixing capacity of numerous batches of the purified material has been determined (*38*). Considering the relative crudeness of the method used, the results have been fairly uniform and have been of much value in the standardization of the purified virus preparations.

For the hemolytic system, sheep red-blood cells were washed four times and suspended in 0.9 percent NaCl solution in concentration of 5 percent. Rabbit-antisheep-cell immune serum was diluted with 0.9 percent NaCl solution to contain 2 units of hemolysin per 0.2 cc. The washed sheep cells were sensitized by mixing 0.2 cc. of hemolysin with 0.2 cc. of cell suspension. Complement was obtained from guinea pigs, titrated immediately prior to its use and diluted to contain 2 units per 0.2 cc. In all instances except one, the papilloma-virus immune serum used was taken from domestic rabbits. In the one case, the serum was from a cottontail rabbit and was made available to us through the generosity of Dr. J. G. Kidd of the Rockefeller Institute for Medical Research. The complement of these sera was inactivated by heating in closed tubes at 56° C. for 30 minutes. Papilloma virus, purified through five ultracentrifugal cycles was diluted with 0.9 percent NaCl solution to concentrations of 70 to 100 gamma per cubic centimeter. For tests, twofold dilutions were used.

The details of this study have already been reported (*38, pp. 198–199*).

Complement-Fixing Unit of Virus Protein

A summary of the results obtained in 25 experiments extending over a period

of 17 months, was also given in the earlier report (*this volume, p. 200*), in which the respective immune sera, protein preparations, and dates of the experiments are indicated. Antiserum when used in repeated tests over a long period of time was frozen, dried, and stored in a refrigerator. With the exception of material II (*table 2, p. 200*), each batch of protein was studied within 3 days after purification. Protein II, however, had stood, in concentrated state, in the refrigerator at 3°–5° C. for several weeks before its use in experiment 1.

Although the tests were made on the basis of 2 units of complement per reaction tube, the results are considered in terms of "complement-fixation units," or the amount of protein which just bound 1 unit of complement in the presence of optimal quantities of antiserum. Under the standard conditions observed, the amount of protein required to fix 1 unit of complement was 0.95 $\pm$0.06 gamma or $10^{-6.023}$ gm. on an average. The coefficient of variation of individual estimates was $\pm$29.8 percent. This variation in individual experiments was remarkably low considering the method and the biological reagents employed and the fact that these were made up under routine conditions over a long period. The values are not considered to be absolute, since variations in technique may alter the value of the complement-fixation unit, but, with the standard technical procedures employed, it is evident that fairly consistent results, within known limits of variation, may be obtained.

It was noted that the observed variations were not definitely related to the behavior of individual protein preparations or antisera. When the mean values for the various materials were examined by methods appropriate for small samples, the observed differences were not greater

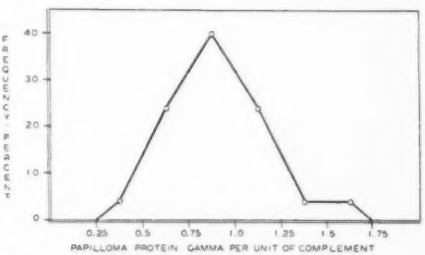


FIGURE 17.—Frequency polygon showing the variation in the quantity of papilloma protein per complement-fixation unit.

than those which could occur due to random sampling. In general the observed differences appear to follow a random distribution as indicated by the symmetry of the frequency distribution shown in figure 17. With the recognized degree of variability, therefore, 1 complement-fixation unit may be defined as 0.95 gamma, or $10^{-6.023}$ gm. of papilloma protein. This quantity of protein in a volume of 0.1 cc., the standard inoculum, would be expected to induce warts after a mean incubation period of 16.9 days.

Relation of Complement-Fixing Unit to Median Individual Effective Dose (50-Percent-Point Dose)

It is of interest to calculate the relation of the complement-fixing unit to the 50-percent-point infectious unit of the protein. As described this unit is, on an average, $10^{-8.355}$ gm., or 0.00441 gamma, of protein. Since for practical purposes the statistical averages may be considered as constant, the ratio of complement-fixation unit to infectious unit may be established on the basis of a criterion common to both infectivity and complement-fixation studies, namely, protein quantity. The ratio of complement-fixation unit to infectious unit is, therefore, 0.95 gamma to 0.00441 gamma, or approximately 215:1. Simultaneous studies of complement-fixing capacity and infectivity on the same pro-

tein material have been made in only a few instances. The ratios in two experiments of this sort were 260:1 and 295:1, respectively. In these experiments, however, the protein values obtained for the complement-fixation units were among the highest obtained, and it is probable that ratios as great as these might be expected to occur less frequently than ratios nearer the mean result of 215:1.

The work reported to this point has been concerned with the purification of the papilloma virus and with the study of the properties of the purified material. Within the limits of the methods available and those developed, efforts have been directed toward standardization and the determination of quantitative relations. Purification of the virus was carried out under conditions which were always as nearly uniform as possible, and the results observed can be interpreted only as applying to material obtained and handled in this way. So long as the standard conditions are maintained, it is possible to correlate physical, chemical, and biological behavior and to feel justified in making quantitative interpretations within the limits established under specified conditions. Thus far infectivity and complement-fixing capacity have been correlated with virus mass and with each other on the basis of virus mass. It should now be strongly emphasized that the relations discovered may apply only under these conditions and cannot be carried over *a priori* for the purpose of interpreting findings under other conditions.

HEAT INACTIVATION OF INFECTIVITY AND COMPLEMENT-FIXING CAPACITY OF PAPILLOMA-VIRUS PROTEIN

Studies have been made on the thermal inactivation of the papilloma protein with respect both to infectivity and to complement-fixing capacity. From investigations with crude extracts, the infectivity of the papilloma virus is known to be relatively heat stable. Shope (*1*) demonstrated the resistance of infectivity to temperature up to 65° C.; actually, the infectivity of extracts heated at 45°–65° for 30 minutes seemed to exhibit a degree of activity higher than that of preparations not heated.

Papilloma protein was purified in the routine way, employing from 4 to 7 ultracentrifugal cycles. The final pellet obtained was dissolved in phosphate buffer pH 6.5 and diluted with 0.9 percent NaCl solution to contain 0.1 mg. protein per cubic centimeter. Aliquot volumes of the preparations (1.5 or 2.0 cc.) were pipetted into long-necked, very thin-walled pyrex bulbs of about 5.0-cc. capacity specially blown from 5 mm. I. D. glass tubing. The bulbs were sealed in a flame leaving the neck about 4 cm. long. A water bath was brought to the desired temperature, and the bulbs, held in a rack, were completely immersed in the water. The water in the bath was agitated by moving the tube rack up and down, and this at the same time served in a small way to stir the contents of the bulbs. At intervals one of the bulbs was taken from the bath and plunged into ice water where it was kept until time for titration. It should be noted that the conditions were not controlled with respect to strict uniformity of salt content or of pH, which was between 6.5 and 7.0.

Test for changes in complement-fixing capacity were made in the usual way by comparing the effect of a control aliquot of virus with that of the respective heated samples. For the infectivity titrations, 12 dilutions of heated and control materials were made in twofold steps with 0.9 percent NaCl solution. Titrations were made in 3 groups of 4 rabbits each. In 1 group,

each dilution of the unheated solution was put in 1 spot in each rabbit and comparable dilutions of 1 heated material were inoculated in similar manner in the same group. In a second group, 2 heated materials were inoculated, and in the third group, the other 2 heated solutions were titrated. Changes were calculated in terms of potency ratio between the effect of the control and that of each heated solution.

The effects of heat on infectivity are summarized in figure 18 in which the curves through the observed points are drawn by inspection. It had been hoped initially that it would be possible to obtain some idea of the nature of the reaction occurring on thermal inactivation. Diminution of infectivity occurs extremely rapidly as shown, and the measurement of infectivity was not sufficiently accurate to make interpretations from this point of view. The observed points as results of the biological measurement of infectivity, nevertheless, indicate clearly the rapid and profound effect of heat even at 50° C. At 50°, 57°, and 60°, inactivitation has proceeded to comparable levels in the first period of 3.75 minutes, while at 63° and 66°, no infectivity remained after this interval. No increase in infectivitv with heating was observed at any

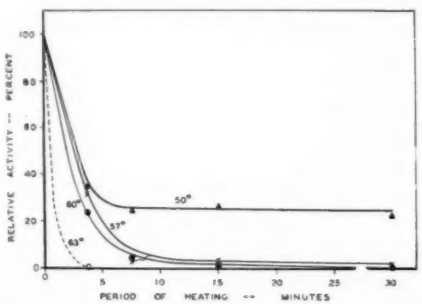


FIGURE 18.—Rate of loss of infectivity associated with the papilloma protein heated at various temperatures.

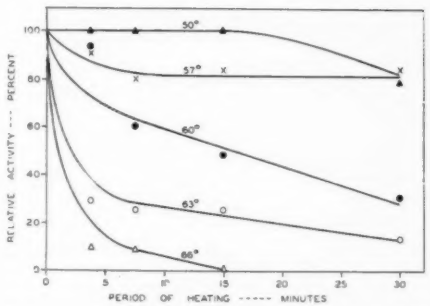


FIGURE 19.—Rate of loss of complement-fixing capacity of papilloma protein heated at various temperatures.

temperature indicating that the apparent increase found with crude extracts (*1*) was apparently a reflection of factors in the extracts other than those referable to the virus itself.

In figure 19 are shown the effects of heat on the complement-fixing capacity of the protein. Here the rate and degree of inactivation at the same temperatures are of an entirely different order from those associated with infectivity. It should be remarked that the particular procedure employed for measuring complement-fixing capacity is not a strictly quantitative one, being subject to relatively wide variations in individual experiments. The interpretations made here should be considered with this in mind.

From the comparison of the points of figure 18 with those of figure 19, it is evident that the rate of thermal inactivation of complement-fixing capacity differs very greatly from that of infectivity. This is better seen in figure 20 where the changes in the two properties at 57° C. are compared. After 3.75 minutes, about 70 percent of the infectivity disappeared, whereas a diminution of only 10 percent in complement-fixing capacity occurred. The difference was still greater after 7.5 minutes when 97.3 percent of the infectivity was lost as compared with 20 percent of

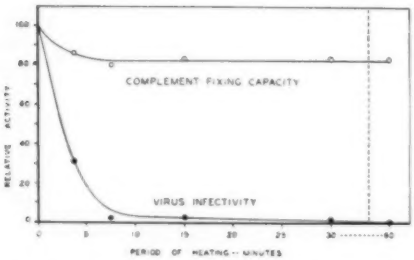


FIGURE 20.—Relative stability of complement-fixing antigen and infectivity of papilloma-virus protein heated at 57° C.

the complement-fixing capacity. In the experiment at 50° no complement-fixing capacity was lost in 15 minutes, but infectivity diminished 75 percent.

Not enough experiments have been made to justify quantitative interpretation on a statistical basis, but the data furnish certain qualitative information. It is clear that the complement-fixing capacity of the protein is not wholly related to the property of infectivity. At 50° and 57° C., the diminution in infectivity is much greater than that in complement-fixing capacity. The greatest difference between the two at these temperatures was about 70 percent. Differences this great were not seen at higher temperatures in the time intervals studied, for after 3.75 minutes at 50° nearly 80 percent of virus infectivity had disappeared and a marked change in complement-fixing capacity had occurred in the same time. The experiments show that only a relatively small part of the complement-fixing capacity is related to that portion of the papilloma complex concerned with infectivity. Taken at face value the results indicate two to three times as much complement-fixing capacity associated with a component or with components of the complex not related to infectivity as that associated with the infectious component of the virus. These findings may be compared with those of Friedewald and Kidd (*39*), who, contrary to earlier results, found an extract heated at 56° to contain a little less virus and a little less complement-fixing antigen, at 60° to have lost most of its infectivity though retaining some part of its ability to fix complement, and at 66° to contain no demonstrable infectivity and only slight power to fix complement.

The papilloma protein is coagulated and precipitated at 65°–66° C., temperatures far above those associated with rapid inactivation of infectivity, but not greatly above the level for inactivation of the complement-fixing capacity. With respect to infectivity and precipitation the papilloma protein behaves toward heat as does the tobacco mosaic virus (*40*) in the instance of which the reaction concerned with diminution in infectivity proceeded with a far greater velocity than precipitation of the protein. As in the instance of tobacco mosaic virus, changes in the papilloma protein leading to final denaturation occur as a series of reactions marked by (1) loss of infectivity, (2) diminution in the antigenic capacity of a portion of the complex not associated with infectivity, and (3) coagulation and precipitation. It is possible that loss in antigenicity with respect to complement-fixing capacity (that occurring after infectivity is lost) is again the result of a series of reactions related to changes in more than one component of the protein molecule. The papilloma complex can be broken down into a number of specific macromolecular components by proper treatment (*17*), but their properties have not been studied.

NEUTRALIZATION OF PURIFIED PAPILLOMA VIRUS WITH IMMUNE SERUM

Mechanisms of the reaction and the relations between virus and antiserum in the

phenomenon of neutralization have been difficult to investigate, primarily because of the lack of suitable materials. In such studies it is necessary that the constituents of the system concerned be susceptible to some sort of assay both before and after mixing. Until recently, viruses were available only in crude mixtures in unknown physical quantities, and methods had not been developed for quantitative biological standardization of such preparations. Measurements of infectivity, the sole unequivocal criterion of virus quantity, were subject to wide recognized variations, and their utilization as quantitative data were limited.

With access to the purified papilloma protein, however, certain of the requirements for the successful prosecution of quantitative studies on the relations involved in the neutralization reaction appear to have been met. The principal reagents concerned *in vitro* are immune serum and virus. The virus can be measured in absolute units of mass by physical or chemical analysis and thus standardized within relatively narrow limits. By means of incubation-period relations, the chemical and physical findings prior to preparation of the reaction mixtures can be correlated with the results of biological estimation of infectivity by means of procedures that are adequate for following the course of virus neutralization. Thus, the participation of the virus can be determined with respect to infectivity and expressed in terms of absolute units referable to virus quantity from the relation established with control-virus preparations not treated with serum. The neutralizing antibody specific for the papilloma protein has not been available in pure form, and data obtainable with whole immune serum cannot be treated like those for the virus. This difficulty, however, does not entirely preclude the possibility of expressing the relations with respect to the immune serum on a quantitative basis. If definite relations could be found to exist between known quantities of virus and relative amounts of antiserum and if the conditions under which the relations were observed were standardized and reproducible, the properties of the immune serum could be described in terms of its relation to the virus which is measurable in absolute units. The relations of virus and antiserum could then be described on a quantitative basis with respect to both reagents.

Statement of the Problem

In the present work certain of the quantitative relations between antiserum and the purified papilloma virus in the neutralization of infectivity have been determined. These studies (*41*) have been carefully restricted to a limited aspect of the general question, and the data have been collected under standard conditions that can be readily reproduced. The general question is stated thus: What are the neutralizing effects of different quantities of a given immune serum in constant volume on different quantities of papilloma virus in constant volume? The immediate objective of the experiments, determined by the methods employed and the nature of the material available, was the search for the interrelations between (1) serum amount in constant volume (actually, serum concentration in terms of dilution), (2) total virus present in constant volume of mixture (total virus concentration in terms of mass per unit volume), and (3) the quantity of the total virus not neutralized by the serum (concentration of free virus in terms of mass per unit volume). The investigations were made on three different systems of serum-virus mixtures.

(A) For a single experiment, a constant volume of a particular dilution of serum was mixed with successive quantities of virus in constant volume.

Thus in one experiment, the effect of the undiluted serum on different amounts of virus was determined; in another, the effect of the serum diluted 1 : 2 on the same quantities of virus was studied, etc.

(B) Constant volumes of successive dilutions of serum were mixed with a constant amount of virus in constant volume. For one experiment, constant volumes of serial twofold dilutions of serum were mixed with a given constant amount of virus; in another, the same dilutions of serum were mixed with another amount of virus, etc.

(C) A given dilution of serum was mixed with a known amount of virus. Serial twofold dilutions of the mixture were then inoculated into rabbits. Control preparations containing identical quantities of total virus without serum were titrated in the same animals, and the various differences between the experimental and control host responses were taken as the measure of the effect of the serum.

Materials and Methods

The results of incubation-period titrations with control purified virus solutions in the absence of antiserum can be assumed to represent a measure of the action of the total virus present, all of which may be considered "free virus." There is no reason to doubt that all of the virus, or total virus, in such preparations of untreated virus is in the free state. It can be stated with assurance, however, that, if all of the virus is not in the free state, the proportion of free to total virus in these preparations is always the same, and measurements of infectivity then can be considered to constitute measurements of the total virus present. This certainty does not exist in the interpretation of results with the serum-virus mixtures. Whether or not the incubation-period response to a serum-virus mixture represents that to an amount of free virus equal to the total amount of untreated virus which yields the same incubation-period response cannot be said now. For the present experiments, however, the assumption has been made that the virus which causes lesions when serum virus is inoculated, whatever its state, has the same specific infectivity as the virus in pure state and represents an actual quantity of free virus equivalent to a quantity of untreated virus giving the same incubation-period response. The amount of free virus in a given inoculum can be calculated from the potency ratio between the serum-virus mixture and the corresponding control-virus inoculum, both of which initially contained identical quantities of total virus. The infectivity of an individual serum-virus mixture may then be expressed in terms of the amount of untreated virus which would be required to produce the same results as the calculated amount of free virus. The difference between the free virus and the total amount of virus contained in a serum-virus mixture is a measure of the amount of virus neutralized.

Since a fairly large group of experiments has been necessary for the present study, it has not been practicable to employ more than four to six test animals in the individual experiments. Variations in group response have, therefore, been compensated for by titration of a control series of inocula along with the experimental series in the same group of animals used for a given experiment. The unit observations in the various experiments may be expected to show greater variability than would have been obtained with larger numbers of test animals. However, the purpose of this study has not been to establish definite values within known limits of error, but to gain some information as to the trends inherent in the experimental results, and to establish the best approximation to the fundamental relations as a basis for further experimental analysis.

Two antisera were used in this study, D. R. 848 and D. R. 496. Each was obtained from a single domestic rabbit bearing experimentally induced papillomas. The former serum was kept in its original

state in a refrigerator during the course of the experiments, some of which were several months apart. The latter had been frozen and dried *in vacuo*. The sera were inactivated with respect to complement by heating at 56° C. for 30 minutes.

In all experiments the total volume of a given serum-virus mixture was 0.75 cc. Of this volume, 0.3 cc. was serum of the desired dilution, 0.3 cc. was virus in the desired concentration, and 0.15 cc. was 0.9 percent NaCl solution used to wash the inner rim of the small tubes employed before mixing the contents by means of a capillary pipette. The concentration of the virus solution to be added in each case was adjusted so that the final concentration of virus in the total volume of the mixture, 0.75 cc., would be of the desired pI. In this way it is possible to compare animal response to control-virus preparations of known pI with the response to serum-virus mixtures of the same pI or total virus concentration. Since the numerical expression of serum dilution designates only relative antibody amount, the pD value of the serum in a mixture represents the dilution of which 0.3 cc. was added to the mixture and not the final dilution of the serum after addition to the mixtures. Control inocula were prepared by diluting the stock-virus solutions to the appropriate pI values with 0.9 percent NaCl solution.

All mixtures and controls were prepared in parallel and allowed to stand at room temperature (24°–26° C.) for 2 hours. At the end of this period, the tubes containing them were immersed in an ice bath where they were kept during the course of animal inoculation, which required 1–2 hours, depending upon the number of animals used.

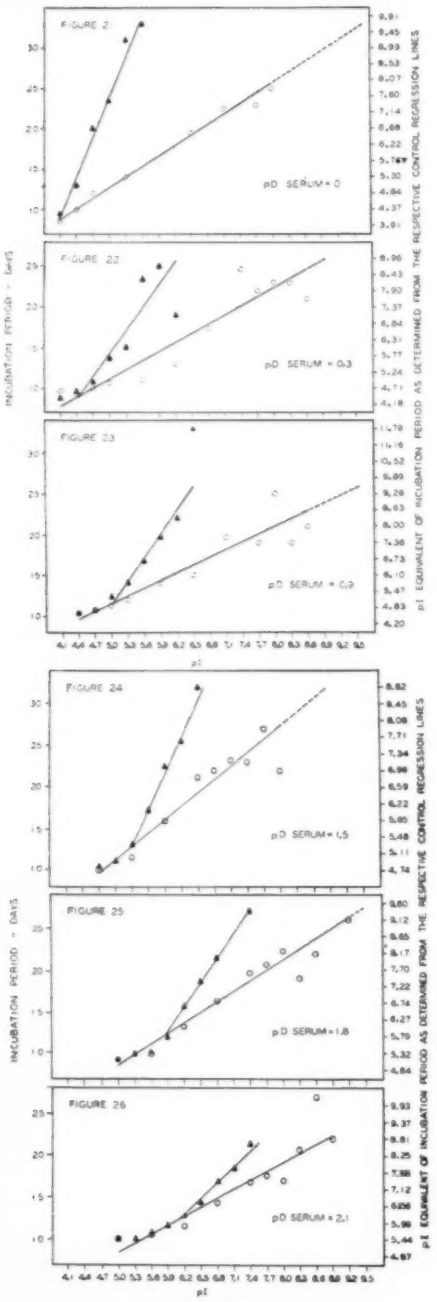
Inoculation of rabbits was carried out according to the procedures described above. In each experiment, the control series of preparations was inoculated on one side of the midventral line, and the serum-virus mixtures were inoculated on the other side of each rabbit in the group. Only one area per rabbit was inoculated with any given inoculum.

Relations Observed When Constant Amounts of Immune Serum D. R. 848 Were Mixed with Successive Quantities of Virus

Nature of the relation between free virus and total virus

Preliminary experiments showed that when successive quantities of immune serum were added to a constant quantity of virus (system B), the relation observed between serum pD and the pI of free virus was not linear, but curved. On the other hand, when constant serum was added to successive quantities of virus (system A) the relation between the variables free virus and total virus appeared to be a straight line for a given dilution of serum. Most of the experiments, therefore, were made with system A to provide a simpler response curve for analysis. The results of experiments made with systems B and C were then tested to learn whether or not the same relations held for all.

Six experiments were made on system A using serum D. R. 848, in each of which a constant volume of a particular dilution of the serum was mixed with successive twofold dilutions of virus. The set-up of these experiments and the results obtained in them are shown in figures 21–26. The serum dilution employed in each experiment is indicated as a pD value in the body of the corresponding figure. Virus concentrations are given in terms of pI values in the abscissas of the figures. These pI values express the total concentration of virus present in the various serum-virus mixtures and in the corresponding control solutions. The left ordi-



nate scale of each figure is employed for indicating the level of the observed mean incubation-period responses to the mixtures and to the corresponding control solutions of pI given in the abscissas. The circles represent average incubation-period responses of all rabbits in a group to the various control inocula containing no immune serum. The solid triangles represent responses to serum-virus mixtures. It will be noted that the pI values in the abscissas are the same in all figures. This standardization was possible because the virus in various purified batches could be assayed by turbidity measurement and diluted for each experiment to give these standard pI values. All figures were thus constructed on the same scale standard with respect to total virus in the mixtures and controls. In this way, the differences in the results of the various experiments with respect to the influence of total virus can be seen by inspection in comparing the figures one with another. Because of lack of space on the test animals, inocula of all pI values in a given control series were not given, but those used, indicated by the circles, were spaced so as to obtain a good estimate of the regression line. In the case of serum-virus mixtures, however, inocula of all pI values to the right of the first triangle appearing in each figure

FIGURES 21–26.—Experimental and control regression lines of experiments of system A, serum D. R. 848. In each figure, the solid triangles show the observed incubation-period response to successive quantities of total virus mixed with constant amounts of the particular dilution of serum D. R. 848 indicated. The circles show the incubation-period responses to the same quantities of total virus to which no serum was added. The respective regression lines were drawn through the points by the method of least squares. pI equivalents of the observed incubation-period response, calculated from the control regression line through the circles, are shown on the right ordinate scale.

were given. The pI values to the right of that corresponding to the last triangle in each figure indicate those dosages which gave no response in the test animals.

It will be observed on inspection of figures 21–26 that, within a range characteristic in each figure and thus depending upon the concentration of serum used in the individual experiments, the incubation period of each mixture, represented by the solid triangles, is increased above that of the corresponding control preparation containing the same amount of total virus. This increase in the incubation period signified a diminution in the infectivity of the individual serum-virus mixture caused, presumably, by the neutralization of some of the virus by the antiserum. Since the average reaction of the group of test animals will be common to all mixtures in a given series as well as to the control virus material, we may gain some idea of the degree of the effects of a given amount of serum on the different amounts of virus in the characteristic range by comparing the incubation-period response to each mixture with that to the control having the same pI. This difference between the response to the individual serum-virus mixture and that to its corresponding control preparation may be employed to determine the potency ratio between the two. Assuming that the unneutralized virus responsible for the biological response to a serum-virus mixture is equal in amount to the quantity of pure untreated virus which would be required to give the same result, the potency ratio between test and corresponding control material having the *same pI* may be determined by use of the equation

$$(7) \quad \text{Log}\left(\frac{\text{Potency test}}{\text{Potency control}}\right)=\frac{y'_2-y'_1}{b}$$

Here y'_1 and y'_2 represent mean incubation period responses to the control and test materials, respectively, and b is the slope of the control response curve determined from the whole series of control inoculations.

It will be observed, however, that the individual points representing response to either serum-virus mixture or control preparation vary rather widely and that a difference between any test mixture and its corresponding control inoculum consequently is subject to considerable experimental error. A better estimate of the potency ratio may be obtained, therefore, by use of the calculated value of y' which represents a point on the control regression line derived from the data as a whole, rather than the more variable observed response to the individual control dosage under consideration. Further, instead of expressing the neutralizing effect of serum in terms of potency ratios between treated and untreated virus, the unneutralized virus of a serum-virus mixture may be considered as free virus having the same specific infectivity as untreated material and expressed as a pI value corresponding to the amount of untreated virus which would be required to give the same incubation-period response. This pI equivalent of the incubation-period response may be calculated simply by substituting the observed incubation-period response to a serum-virus mixture in the equation for the control-response curve obtained with the same group of experimental animals and solving for pI. This may be illustrated with the data of the first experiment in the series (fig. 21) which is given in detail in table 10. By the method of least squares the equation for the control regression line, or control response curve, was found from the data of table 10 (A), to be

$$(8) \quad \text{Incubation period}=-8.966+4.335\,(pI).$$

The incubation period responses to the serum-virus mixtures of this experiment

TABLE 10.—*Data of experiment of figure 21 showing results of control series, A, and serum-virus mixture, B*

Control series (A)				Serum-virus mixtures (B)					
pI of control virus [1]	Areas inoculated	Areas developing lesions	Incubation-period response [2]	pI of total virus in mixtures [3]	pD of serum in mixtures	Areas inoculated	Areas developing lesions	Incubation-period response [4]	pI equivalent of incubation-period response [5]
	Number	*Number*	*Days*			*Number*	*Number*	*Days*	
4.1	4	4	8.5	4.1	0	4	4	9.5	4.26
4.4	4	4	10.0	4.4	0	4	4	13.0	5.07
4.7	4	4	12.0	4.7	0	4	4	20.0	6.68
5.0	0			5.0	0	4	4	23.5	7.49
5.3	4	4	14.0	5.3	0	4	1	31.0	9.22
5.6	0			5.6	0	4	2	33.0	9.68
5.9	4	4	16.0	5.9	0	4	0		
6.2	0			6.2	0	4	0		
6.5	4	4	19.5	6.5	0	4	0		
6.8	0			6.8	0	4	0		
7.1	4	4	22.5	7.1	0	4	0		
7.4	0			7.4	0	4	0		
7.7	4	1	23.0	7.7	0	4	0		
8.0	4	1	25.0	8.0	0	4	0		
Total	36	30				56	19		

[1] Weighted mean $(\bar{x}_1)=5.590$.
[2] Weighted mean $(y'_1)=15.27$.
[3] Weighted mean $(\bar{x}_2)=4.700$.
[4] Weighted mean $(y'_2)=19.00$.
[5] Weighted mean $(\bar{y}_2)=6.451$.

are shown in table 10 (B), column 5. The pI equivalents of these responses, as calculated from equation 8 are given in column 6 of the same section of this table. Using the response in the first row as an example and substituting in equation 8,

(9) $9.5=-8.966+4.355\ (pI)$, and $pI=4.260$,

which is the pI equivalent of 9.5 days, or the amount of free virus in this mixture. Free virus may, therefore, be expressed directly as a pI value, and the difference between this value and the pI of the total virus in the mixture (cf. column 1 of table 10 (B)) represents the logarithm of the potency ratio between the mixture and the corresponding control material.

The above procedures are applicable in all three types of experiments (i. e. systems A, B, and C, described above) and in their application the test with each serum-virus mixture is considered as a single dosage assay on an unknown material, regardless of the trend of responses to the various mixtures in a given series constituting an experiment.

Still greater accuracy would be obtained in the analysis of results if a calculated response curve could be determined for a series of serum-virus mixtures. This would make possible the use of points on a curve determined from a series of observations instead of the more variable individual estimates. Points on the experimental curve could then be compared with corresponding points on the control response curve. As pointed out above, there is an apparently linear deviation of the test responses from those of the control inocula in experiments of system A as may be seen in figures 21–26; that is, when various amounts of virus are mixed with a constant amount of a given dilution of serum, the incubation-period responses are apparently linearly related to the pI of the total virus in the mixtures. The analysis of results would be simpler if the relation could be assumed for all practical purposes to be linear. By in-

spection of the experimental results of figures 21–26, the solid triangles which differ from the control response shown by the circles in each instance are seen to fall fairly well about the best straight line drawn through them by the method of least squares.

It should be noted here that the frequency of lesions is lower with the smaller amounts of virus (higher pI values) and that the terminal points in this region of response in the figures are more variable, but have less weight in determining the slope and position of the regression lines than the other points. In most of the figures, the solid triangles on the extreme right represent only a single response in a single rabbit rather than an average response of 4 to 6 rabbits. This was taken into consideration in drawing the lines by giving proper weight to each point. The lines of figures 21–26, thus drawn, were compared with the observed points by means of the χ^2 test. The probability, P, for goodness of fit as determined by this procedure was 0.94, or greater, in 5 of the 6 experiments. In the experiment of figure 22, P was 0.70, indicating that a worse fit than that observed might occur 70 times in 100 by chance alone. We may assume, therefore, that for a given dilution of serum the relation between pI of free virus and pI of total virus is linear and that we are justified in using points on the calculated experimental regression line for the purpose of comparison with corresponding points on the control regression line.

Before proceeding with the analyses, several points of significance for subsequent discussion should be noted in relation to the two regression lines now established for each experiment. It is seen that considerable variation occurs in the slopes of the control regression lines drawn mathematically through the circles in the various figures. The extent of this variation, however, was no greater than could have occurred by chance alone in individual experiments employing the small numbers of animals. The mean control regression coefficient of this group of six experiments was 4.069, which does not differ significantly from the slope of the previously determined control or standard response curve, namely, 4.096 ± 0.26. The positions of the control regression lines in relation to the left ordinate (incubation period) likewise show considerable variation in comparison with the position of the previously determined standard-response curve. These variations in slope and position, because of the small numbers of test animals, do not interfere with analysis of differences in test and control materials in the individual experiments which involve a common group of test animals. They do, however, prevent a direct comparison of the results of the different experiments involving different groups of test animals on a basis of the incubation-period response *per se*. This difficulty, nevertheless, may be surmounted by the simple expedient of converting the observed serum-virus mixture incubation-period responses to pI equivalents determined from control-virus titrations in each group of animals as described above. These equivalents of the observed incubation periods for the serum-virus mixtures are given in the ordinate scales on the right sides of figures 21–26. By this conversion, the relations between free virus and total virus in the serum-virus mixtures in one experiment can be compared on a quantitative basis with those of the other experiments.

As already emphasized, the experimental regression line was drawn only through those points (solid triangles) which deviated from the control regression line. It is obvious from figures 21–26 that below

certain pI values (i. e., above certain virus concentrations) the results obtained with the serum-virus mixtures coincide with those obtained with the untreated virus. Responses to the mixtures which deviated from the control responses did so in linear fashion. They followed a steeper slope than did those to the control materials, and since the relation is linear, the two regression lines must intersect. Since the amount of free virus in the mixtures cannot be increased above the total amount by the addition of serum, the point of intersection of experimental with control regression line indicates a limiting pI value at and below which no detectable influence of the serum is observed. Beyond this limiting amount of virus, therefore, the results with the mixtures and control preparations would be expected to continue to be identical if the amount of free virus of the former is the same as the total virus of the latter. This result indicates that for any given amount of serum within the range of pI values covered by this study, there is a limiting virus concentration above which the serum is incapable of causing a measurable reduction in infectivity.

The observed regression line of the mixtures is also limited at its other extreme. That is, for a given amount of serum, there is a virus concentration below which not enough active or free virus remains to produce lesions in the rabbit skin. Again the sensitivity of the method may influence the determination of this virus concentration, but for practical purposes the virus may be considered to be completely neutralized when not enough remains to give detectable lesions in the rabbit skin. Theoretically, the lower limit to the calculated regression line is zero virus (or infinite pI), and complete inactivation would be realized only when an infinitely small (i. e., zero) amount of virus is added to the antiserum in the various experiments. Since virus quantities less than that amount required to produce the 50-percent response are beyond the range practical for investigation by infectivity measurement, however, the 50-percent point may be chosen arbitrarily as the level of complete inactivation. In the present experiments, therefore, the 50-percent point of the serum-virus mixture is considered as the average practical limit of infectivity at the lower extreme of virus concentrations (high pI values).

The equation for the experimental regression line

The regression equation relating incubation period response to serum-virus mixtures of pI of the total virus in the various mixtures may be determined in the same manner as for untreated virus preparations. However, as already pointed out, the pI equivalent of the incubation period response, determined by simultaneous titrations of standard virus material in each group of animals, is more accurate for the comparison of results obtained in different experiments than incubation period *per se*. These transformed values of the dependent variate, may, therefore, be used instead of incubation periods in the regression equations. Letting y represent the pI equivalent of a given incubation-period response, or free virus in terms of pI, and x the pI of total virus in the mixtures, the linear regression of y on x is given by the straight line equation $y=a+bx$, where a and b are constants (within limits of random variation) for any given amount of serum (i. e., constant serum pD).

For a given set of data determined by experimentation, the procedure discussed by Fisher (*42*) may be employed, and the regression equation used in the form

$$Y=a+b\,(x-\bar{x}) \qquad (10)$$

where $\bar{x}$ is the mean concentration of total virus (i. e., pI) of the series of serum-virus mixtures which show a measurable reduction in activity and where the two parameters a (the position constant in relation to the ordinate) and b (the regression coefficient or slope) are to be estimated from the data at hand. Y is used to represent calculated values of y or free virus. In this case a is simply the mean, $\bar{y}$, of the observed values of the independent variate, the pI equivalent of free virus, obtained in the experiment. Therefore,

$$a=\bar{y} \tag{11}$$

and equation 10 may be written

$$Y=\bar{y}+b\ (x-\bar{x}) \tag{12}$$

where Y is the predicted value of y (cf. Fisher (*42*)) or free virus for each value of x of total virus.

The neutralization data of the experiment of figure 21 and table 10 (B) may be used to illustrate the use of this equation. The means, $\bar{x}$, and $\bar{y}$, are seen to be 4.700 and 6.451, respectively, while, b, by standard statistical procedures (*42*), is 3.778. Substituting these values in equation 12, equation 13

$$Y=6.451+3.778\ (x-4.700) \tag{13}$$

is obtained for the relation of the pI of free virus to pI of total virus in this particular experiment. Therefore, when total virus x is mixed in various amounts with a definite and constant amount of serum, as pD 0 of serum D. R. 848, the amount of free virus may be calculated from the above equation by substituting a known value of x and solving for Y in each case.

Relation of total virus to amount of virus neutralized by pD 0 of antiserum D. R. 848 in experiment of figure 21

Since the total virus x is known and the free virus y remaining after addition of serum can be determined by experiment, the difference between these actual amounts will represent the virus neutralized. The values of x and y represent logarithms of the reciprocals of actual virus amounts (i. e. pI). Therefore, the difference between the respective virus amounts must be determined from their antilogs as in the equation

$$\Delta=10^{-x}-10^{-y} \tag{14}$$

where Δ is the amount of virus neutralized, in grams per 0.1 cc. Figure 27 shows the relation of the amount of virus neutralized to the total amount of virus mixed with pD 0 of serum D. R. 848. In constructing this figure the quantities of free virus expected from the trend of the data as a whole (i. e. values of Y determined from equation 13) were employed instead of the more variable observed values (i. e., y) shown in the last column of table 10. The values in this figure are shown in gamma per 0.1 cc. The actual amount of antibody used in this experiment is not known, but the relative concentration bears a definite relation to the actual amount and can be converted eventually if it becomes possible to determine the antibody content of serum D. R. 848. The curve of figure 27 does, however, represent the nature of the relation between the amount of virus treated and the amount neutralized for a given dilution of serum. Similar curves were ob-

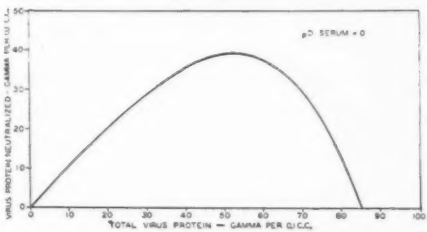


FIGURE 27.—The relation of the amount of virus neutralized to the total amount of virus mixed with undiluted (pD=0) serum D. R. 848.

tained with the different antibody concentrations (serum dilutions) used in all six experiments though the constants of the reaction were, of course, different in each case.

Significant interpretations can be made from the curve of figure 27. For any given amount of antibody there is a certain absolute quantity of virus, that will be neutralized by it. On increasing the amount of virus mixed with this given quantity of serum the absolute amount of virus neutralized is decreased, and a virus concentration is ultimately reached at and above which no detectable effect is exerted by the serum. This finding, discussed at greater length in a subsequent paragraph, is similar to that relative to the "inhibitory effect" of excess antigen in *in vitro* antigen-antibody reactions involved in the precipitin reaction (*43*). Obviously, virus concentrations below that which gives maximum neutralization will show a decrease in the absolute amount neutralized as the quantity of virus added to the antibody is decreased though the proportion neutralized is systematically increased. The curve of figure 27, describing the neutralization of virus by a constant quantity of antibody, therefore, has two limits and a maximum.

Trends in experimental results associated with progressive decreases in antiserum concentration

Further analysis of the data of figures 21–26 shows that the slope of the experimental regression line decreases with successive dilutions of serum and that the point of intersection of control and experimental lines trends to the right as the concentration of serum is decreased (i. e. as the pD of the serum is increased). This is better illustrated in figure 28 in which the experimental regression lines of figures 21–26 have been entered simultaneously using the observed pI equivalents of free virus, y, and total virus, x, as coordinates; the light broken lines represent expected regression lines which will be discussed below. The points of convergence of control and experimental regression lines, hereafter referred to as coincidence points, are represented by the intersections of the regression lines of figure 28 with the lower diagonal line. These intersections are the points at which the amount of free virus after mixture with serum is the same as the total virus, or at which no neutralization of virus was observed. The solid circles represent, for each regression line, the coordinate points, $\bar{y}$ and $\bar{x}$, which are the means described above, that is, mean free virus and mean total virus, respectively, of a given set of experimental data. The points marked + on the regression lines represent the respective 50-percent points of the serum-virus mixtures in the various experiments. The 50-percent points, +, though widely scattered fall at random about a horizontal line drawn through the mean value for the entire group of experiments, namely, $y=$pI 8.368. In spite of the wide fluctuations of the individual 50-percent-point estimates seen here, the mean of the group is remarkbly close to the average value found for the 50-percent point (designated as pI_{50}) in studies on standard virus preparations described above, namely, $pI_{50}=8.355$. If the concept of the stability of the 50-percent point of standard virus preparations is correct, we should expect the 50-percent points of the present series of experiments to lie, as they apparently do, about a straight horizontal line drawn near the level, $y=$pI 8.355.

The coincidence points must, by nature of the graph, fall along a line with a slope of unity and pass through zero coordinates since they represent points at which $y=x$. The fact that the level of intersection is progressively increased as the concentra-

tion of serum employed for the series is decreased (pD is increased) indicates a correlation of the coincidence points with serum pD. Similarly, the group means represented by the solid circles of figure 28 follow a definite trend upward as serum pD is increased. It appears, therefore, that this statistic is likewise correlated with serum pD. Finally, the progressive decrease in slope of the experimental regression line indicates a correlation between slope and serum pD.

In order to test the significance of the above trends, the data have been grouped according to serum pD (table 11 (A)), and the correlation coefficients determined for the respective statistics in relation to serum pD. The correlation coefficients are given in table 11 (B) together with the probabilities that the observed correlations could be due to chance alone. The following notations are used in the table and in subsequent discussions:

x_{50}=Total virus in the mixture which yields 50-percent positive and 50-percent negative inoculation sites (in terms of pI).

x_e=Total virus in the mixture at the point of coincidence of control and experimental response curves (in terms of pI).

$\bar{x}$=Total virus in the mean of a given series of mixtures the response to which deviates from the control response (in terms of pI).

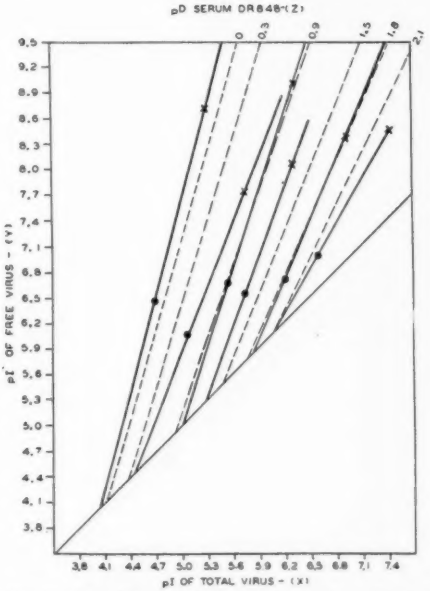


FIGURE 28.—Experimental regression lines (heavy continuous lines) of the experiments of figures 21–26 (serum D. R. 848) compared directly on the basis of pI equivalents of the observed incubation-period response. (See text.) The symbol + indicates the observed 50-percent points (y_{50}, table 11), and the solid circles indicate the coordinate points $\bar{y}$ (mean of free virus) and $\bar{x}$ (mean of total virus) for each regression line. The broken lines indicate responses expected for the various dilutions of serum according to the relations described in equation 19.

TABLE 11.—*Correlation of statistics with dilution of serum in experiments with antiserum D. R. 848*

Statistic[1]	A						B		
	pD of antiserum D. R. 848						Correlation of statistic with serum pD		
	0 (Exp. 1)	0.3 (Exp. 2)	0.9 (Exp. 3)	1.5 (Exp. 4)	1.8 (Exp. 5)	2.1 (Exp. 6)	Correlation coefficient (r)	Probability (Fisher's table V A) (P)	Remarks
x_{50}	5.3000	5.7400	6.3141	6.3128	6.9470	7.4500	0.961	<0.01	Significant correlation.
x_e	4.0695	4.4764	5.0210	5.2897	5.8518	6.0992	.990	<.01	Do.
$\bar{x}$	4.7000	5.0750	5.5555	5.7579	6.2200	6.5964	.985	<.01	Do.
y_{50}	8.7178	7.7114	8.9902	8.0620	8.3701	8.4984	.010	>.10	No correlation.
$\bar{y}_e$	4.0695	4.4764	5.0210	5.2879	5.8518	6.0992	.990	<.01	Significant correlation.
$\bar{y}$	6.4512	6.0412	6.6620	6.5547	6.6982	6.9827	*.796	.060	Probable correlation.
b	3.7777	2.5117	3.0091	2.7163	2.2997	1.7757	−.801	.056	Do.

[1] See notation in text.

y_{50} = Free virus in the mixture which yields 50-percent positive and 50-percent negative inoculation sites (in terms of pI).

y_e = Free virus in the mixture at the point of coincidence of control and experimental response curves (in terms of pI).

$\bar{y}$ = Free virus in the mean of a given series of mixtures the response to which deviates from the corresponding control response (in terms of pI).

b = Slope of a given experimental regression line.

It is to be noted that high correlations exist (1) between serum pD and x_{50} or the pI of the serum-virus mixture which yields 50-percent positive and 50-percent negative inoculation sites, (2) between pD and $\bar{x}$ or the mean pI of the series of mixtures which show measurable decreases in virus infectivity because of the serum, and (3) between pD and x_e ($=y_e$) or the pI at the point at which there is no decrease in virus infectivity due to the serum. It will be noted further, that $\bar{y}$ or the mean amount of free virus, is probably correlated with serum pD. The correlation coefficient ($r=0.796$) is high in this instance, but its significance is somewhat lessened by the large error associated with the small number of observations. The probability $P=0.06$ indicates that a value this high would be obtained by chance alone 6 times in 100. Although the result could be due to chance, it is not very probable, and the chance of being right in the assumption that this statistic is correlated with pD is, therefore, fairly good. This assumption is further justified by the finding of a highly significant correlation of this statistic with pD in the results observed with serum D. R. 496 described in table 12. In a similar way it may be assumed that the correlation between pD and b, the slope of the experimental regression line, is probably significant ($r=-0.801$). The probability $P=0.056$ is about the same as in the foregoing instance, and the assumption that this correlation is real is further supported by a highly significant correlation of similar sort with serum D. R. 496 (table 12). The correlations herein demonstrated may, therefore, be used to determine the quantitative relations between the variables (1) total virus, (2) free virus, and (3) relative antibody concentration in the serum-virus mixtures.

Analysis of the quantitative relations between total virus, free virus, and antiserum D. R. 848

It has been shown that equation 12 may be used to express the relation between total virus and free virus when the amount of serum employed in the mixtures is

TABLE 12.—*Correlation of statistics with dilution of serum in experiments with antiserum D. R. 496*

Statistic[1]	A					B		
	pD of antiserum D. R. 496					Correlation of statistic with serum pD		
	0 (Exp. 10)	0.3 (Exp. 11)	0.9 (Exp. 12)	1.8 (Exp. 13)	2.7 (Exp. 14)	Correlation coefficient (r)	Probability (Fisher's table V A.) (P)	Remarks
x_{50}	5.5310	5.8100	6.3500	7.2400	7.8500	0.998	<0.01	Significant correlation.
x_e	4.0380	4.3280	5.0936	5.9307	6.4760	.992	<.01	Do.
$\bar{x}$	4.7571	5.0656	5.6200	6.6050	7.1600	.992	<.01	Do.
y_{50}	8.3649	7.8348	8.1510	8.4906	8.6616	.734	>.10	No significant correlation.
y_e	4.0380	4.3280	5.0936	5.9307	6.4760	.992	<.01	Significant correlation.
$\bar{y}$	6.1236	6.0734	6.3730	7.4628	7.5640	.945	.0156	Do.
b	2.8963	2.3662	2.4357	1.8860	1.5907	−.987	<.01	Do.

[1] See notation in text.

constant and where the values $\bar{x}$, $\bar{y}$, and b are determined from the experimental data at hand. Equation 12 is repeated here:

$$(12) \qquad Y=\bar{y}+b\ (x-\bar{x}).$$

As shown in the preceding discussion, the values of $\bar{x}$, $\bar{y}$, and b actually observed in the experiments of figures 21–26 varied systematically with systematic changes in the relative concentration of serum D. R. 848. It has been assumed that the respective correlations with serum pD are linear, within the range of serum concentrations covered by the present observations, for no systematic departure from a linear relation could be detected with the data at hand. It is possible, therefore, to express each of these variates as a function of serum pD using the equation for the regression of each variate on pD as calculated from the data of table 11 (A). Thus it is found that

$$(15) \qquad \bar{y}=6.2444+0.2914z,$$

$$(16) \qquad \bar{x}=4.7458+0.8227z, \text{ and}$$

$$(17) \qquad b=3.4084-0.6516z$$

where z represents the pD of serum employed in the mixtures and the other notations have their previous significance.

By substituting the expressions on the right of equations 15, 16, and 17 for $\bar{x}$, $\bar{y}$, and b, respectively, in equation 12 a relation is obtained which includes the three variables, total virus, free virus, and antiserum, namely,

$$(18) \quad Y=(6.2444+0.2914z)\ +(3.4084 -0.6516z)[x-(4.7458+0.8227z)].$$

This, when simplified, becomes

$$(19) \quad Y=0.5361z^2-0.6516zx+0.5788z +3.4084x-9.9312,$$

where Y is the calculated value of y (free virus) for any combination of x (total virus) and z (serum). It should be remembered that the values of x, Y, and z in this equation are in terms of logarithms of reciprocals as represented by pI and pD.

Equation 19 represents the best approximation of the relations between total virus, free virus, and serum D. R. 848 as determined statistically from the present series of observations. It is recognized that the statistical variables in this analysis are subject to random errors associated with biological experiments when small numbers of animals are employed for the unit observations. It has not been the purpose of this study to estimate the true values of the constants of equation 19, i. e., those which would be obtained with an infinite series of animals, but to obtain some information as to the nature of the relation between the three variables as a basis of approach to further quantitative studies. The equation can be considered strictly applicable, therefore, only within the limits of the x and z values included in this study and then with the recognition of random variations inherent in the method.

The expected response curves, referred to above, for the amounts of virus and serum employed in the experiments of figures 21–26 have been calculated from equation 19. They are represented by the broken lines of figure 28. It will be noted that two of the six experimental regression lines are almost identical with the expected while the remaining four vary about their respective calculated counterparts in random fashion. In all of the experiments the χ^2 test for goodness of fit gave a probability, P, greater than 0.10. For these χ^2 tests, the expected results (Y values calculated from equation 19) were transformed into incubation-period equivalents on the basis of the control response for comparison with the observed incubation-period responses.

We may conclude, therefore, that equation 19 expresses satisfactorily the relations between total virus, free virus, and antiserum, and that interpretations based on this equation will not differ significantly from those based on the actual observations within the limits of virus and serum concentrations employed in the present study.

Experiments in Which Constant Total Virus Was Mixed With Successive Dilutions of Antiserum D. R. 848

Two experiments were made in which a constant quantity of papilloma virus was mixed with each of 12 successive twofold dilutions of antiserum. In the first of these experiments, the results of which are shown

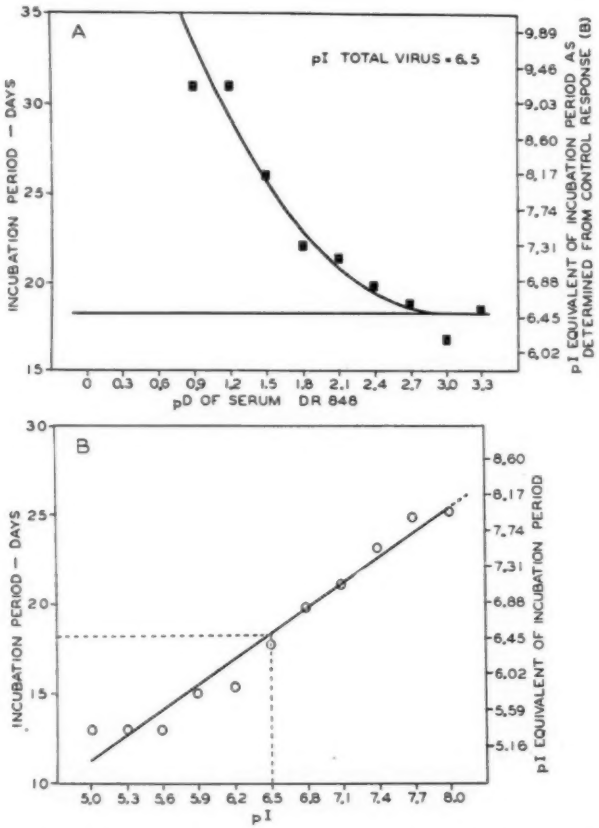

A
pI TOTAL VIRUS = 6.5
INCUBATION PERIOD — DAYS
pI EQUIVALENT OF INCUBATION PERIOD AS DETERMINED FROM CONTROL RESPONSE (B)
pD OF SERUM DR 848
B
INCUBATION PERIOD — DAYS
pI EQUIVALENT OF INCUBATION PERIOD
pI

Figure 29.—Incubation-period response to a constant amount of total virus (pI 6.5) mixed with successive dilutions of serum D. R. 848. *A*, Points show observed response. The curve was drawn by inspection. Horizontal line represents level of response to untreated virus of pI 6.5 as determined from control regression line of *B*. *B*, Control regression line of untreated virus in same group of test rabbits.

in figure 29, the amount of virus employed was $10^{-6.5}$ gm. per 0.1 cc. of the serum-virus mixture, i. e., pI 6.5. This experiment was carried out in the early course of the present study and the apparent tendency for the incubation-period response to curve in this case led to direction of interest to the previous type of experiment, that is, with constant serum, in which the response was linear and therefore simpler to analyze. The control regression line of figure *29, B*, was used to determine the pI equivalents of the incubation-period responses in this group of animals in the same manner as that described in the foregoing analyses. Thus, the free virus in the mixture of figure *29, A*, could be determined from the respective incubation-period responses

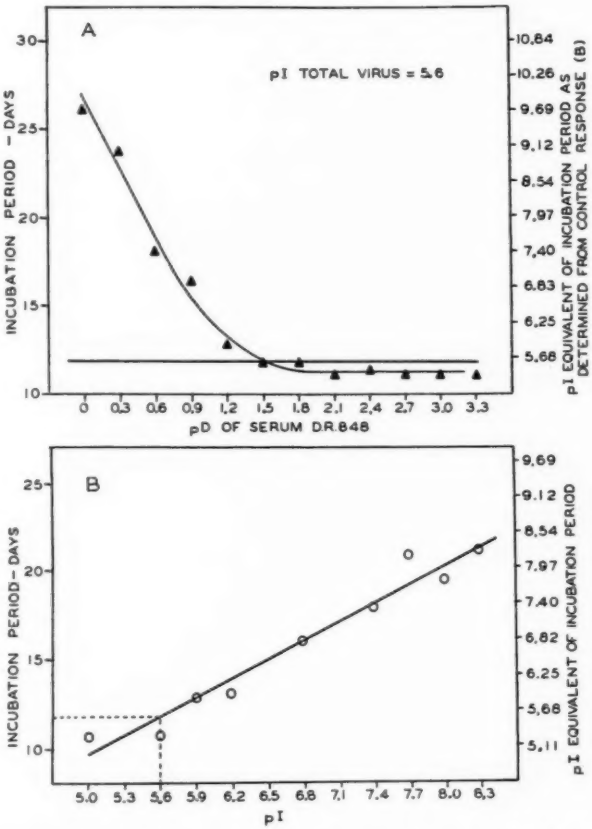

A
pI TOTAL VIRUS = 5.6
INCUBATION PERIOD - DAYS
pI EQUIVALENT OF INCUBATION PERIOD AS DETERMINED FROM CONTROL RESPONSE (B)
pD OF SERUM D.R.848
B
INCUBATION PERIOD - DAYS
pI EQUIVALENT OF INCUBATION PERIOD
pI

FIGURE 30.—Incubation-period response to a constant amount of total virus (pI 5.6) mixed with successive dilutions of serum D. R. 848. *A*, Points show observed response. Curve was drawn by inspection. Horizontal line represents level of response to untreated virus of pI 5.6 as determined from the control regression line of *B*. *B*, Control regression line of untreated virus in same group of test rabbits.

by converting them to the pI equivalents, which are given in the right ordinate scale on the basis of the control response in the same group of animals. Since the experimental response is apparently curved, no attempt has been made to predict its exact nature from the limited data of this experiment alone. The curve through the solid squares in figure *29, A* was drawn by sight. Since an equation has been found which describes very well the quantitative relations of the previous section, we may now substitute the total virus, x, and the serum amounts, z, used in the present experiment in equation 19 to ascertain whether it also serves to predict results obtained with a constant amount of virus and variable quantities of antiserum. The results shown in figure *29, A*, have been converted to pI equivalents of free virus and plotted against total virus in the upper curve of figure 31. In this figure the continuous line, drawn mathematically through the solid squares by substitution in the equation, represents the expected response from equation 19, using the constant value pI 6.5 for x, and the various z values shown along the abscissa in terms of serum pD. The solid squares represent the observed free virus as determined from observed incubation-period responses. Although the calculated response does curve slightly the curvature is less than that which would best fit the observed points. Nevertheless, it is obvious that the observed points correspond very well to the expected. When the expected responses are converted from pI equivalents to incubation-period values expected from a group of animals which reacts to the control-virus preparations as in figure *29, B*, the χ^2 test for the goodness of fit gives a probability, P, of 0.99. Thus, it is seen that equation 19 predicts well within limits of random sampling the results to be expected in the present experiment.

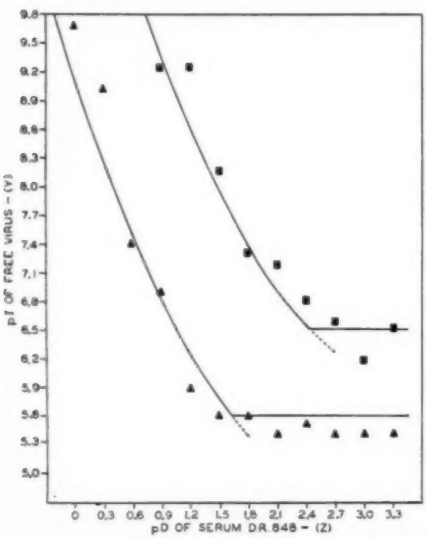


FIGURE 31.—Relation of free virus in terms of pI equivalents to serum pD when successive dilutions of serum are added to constant quantities of total virus. Solid squares represent observed points of the experiment of figure 29, and solid triangles those of the experiment of figure 30. The respective curves, drawn mathematically by substitution of the observed data in equation 19, represent responses expected.

In the second experiment of this type a constant quantity of virus corresponding to pI 5.6 was employed. The experimental and control results are shown in terms of incubation-period response in figure 30, *A* and *B*, respectively. The converted observed response in terms of pI equivalents of free virus plotted against serum pD is shown in the solid triangles in figure 31, as compared with the expected response indicated by the continuous line of the lower curve. Again the fit is excellent as determined by χ^2, based on observed and calculated incubation-period response, since $P=0.99$.

The results confirm the relations between total virus, serum, and free virus as described by equation 19. It should be recalled that the equation is limited at the

point at which free virus is equal to total virus since there can be no increase in virus due to the addition of serum. Beyond this point all mixtures will give the same response as the control preparations of the same pI, and for constant amounts the response will be a horizontal line (fig. 31) through the ordinate at the level of the pI corresponding to the total virus concentrations.

Results Obtained When a Mixture of Papilloma Virus and Antiserum D. R. 848 Was Diluted Successively and Inoculated into Rabbits

In this experiment a mixture of serum D. R. 848 in the 1:4 dilution, pD=0.6, and virus in a final concentration of pI 5.0 was prepared. The mixture was allowed to stand at room temperature (24°–26° C.) for 2 hours and then carried through 12 successive twofold dilutions with 0.9 percent NaCl solution. Each dilution of the mixture, as well as each of a series of control virus dilutions, was inoculated into a group of test animals. The average response of the rabbit group to control inocula is shown by the circles of figure 32. The responses to the various dilutions of the serum-virus mixture are shown by the solid triangles of figure 32. The pI scale along the lower abscissa gives the virus content of each dilution of the mixture as well as that of the corresponding control preparation. The upper abscissa scale shows the relative amount of antibody, in terms of pD of the original serum, in each dilution of the mixture. The left ordinate scale shows the incubation-period response to control and experimental materials, and the right ordinate scale shows the pI equivalents of the incubation-period responses as calculated from the control regression line drawn mathematically through the circles. As in the previous analyses, the pI equivalent of the incubation-period response is taken as the amount of free virus. Figure 33 shows the observed free virus, y, (solid triangles) plotted against total virus, x. The broken line of this figure represents the control response where free virus equals total virus, and the continuous line represents the expected response to the dilutions of the serum-virus mixture calculated by means of equation 19. Beginning with x=pI 5.0 and z=pD 0.6 for the original serum-virus mixture, each value was increased by 0.3 in each successive twofold dilution of the mixture. Substituting val-

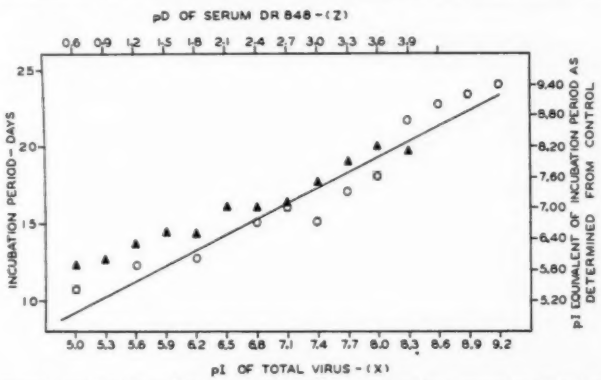


FIGURE 32.—Observed incubation-period response (solid triangles) to successive dilutions of a serum-virus mixture of pI 5.0 and pD 0.6. Circles represent response to control virus solutions. The line indicates the control-regression line for conversion of incubation-period response to pI equivalents.

ues thus obtained in equation 19 and solving for Y in each case, the continuous line curve of figure 33 was obtained. As in the previous analyses, the equation is limited at the point where the free virus gives the same response as the total virus. Beyond this point the response to dilutions of the mixture should coincide with that to the corresponding controls. Whether or not the point of coincidence, as predicted in this case by equation 19, is accurate cannot be stated since the serum quantity involved in the dilution giving this point, namely, about pD 3.0 was outside the limit (beyond pD 2.1) actually covered by experimental observations on which the equation was based. The nature of the correlation between the different variables in this region, therefore, is not definitely known. Regardless of this fact, the fit is excellent as may be seen from inspection of figure 33; this is confirmed by the high probability, $P=0.99$, obtained for the χ^2 test, when observed and expected incubation-period responses are compared.

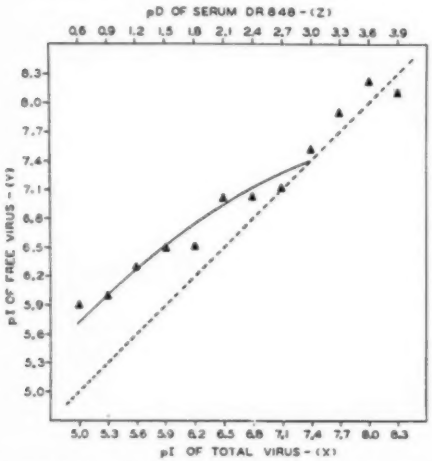


FIGURE 33.—The relation of free virus, in terms of pI equivalents, to serum pD and pI of total virus in the experiments of figure 32. Solid triangles represent observed points. The curve through them represents response expected from equation 19. The broken line represents response to the control solutions of untreated virus.

With dilution experiments of the present type, therefore, equation 19 gives a very good estimate of results to be expected. This close correspondence of observed with expected results further confirms the relationship between serum, total virus, and free virus as expressed by equation 19.

EXPERIMENTS IN WHICH CONSTANT AMOUNTS OF IMMUNE SERUM D. R. 496 WERE MIXED WITH SUCCESSIVE QUANTITIES OF VIRUS

Five experiments using serum-virus mixtures of system A have been made with serum D. R. 496. In each experiment a constant volume of a particular dilution of this serum was mixed with successive amounts of total virus. The relative serum concentrations employed in the different experiments were pD=0, pD=0.3, pD=0.9, pD=1.8, and pD=2.7, respectively. The individual experiments gave results quite similar in type to those obtained with serum D. R. 848 shown in figures 21–26, and, therefore, are not reproduced here. The results obtained in the group of experiments with serum D. R. 496, however, are summarized in the continuous lines of figure 34 and in table 12 (A). The broken lines of figure 34 are expected response curves, calculated from the data described below. It will be seen in table 12 (B) that all but one of the statistics in the first column are significantly correlated with the pD of the antiserum. As pointed out above, this one exception, namely, the amount of free virus in the mixture yielding 50-percent positive and 50-percent negative inoculation sites, y_{50}, would not be expected to be correlated with serum pD. Any material which yields 50-percent positive and 50-percent negative

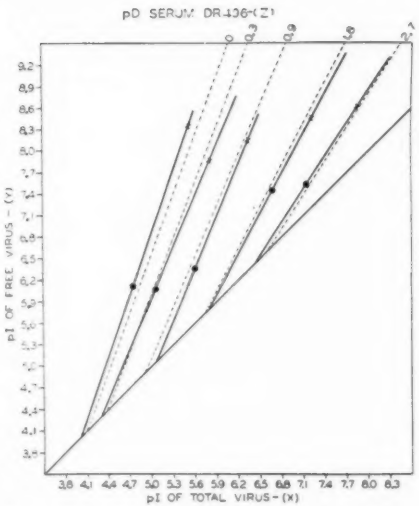


FIGURE 34.—Experimental regression lines (heavy continuous lines) of experiments with serum D. R. 496 compared directly on the basis of pI equivalents of the observed incubation-period response. The symbol + indicates the observed 50-percent points (y_{50}, table 12), and the solid circles indicate the coordinate points $\bar{y}$ (mean of free virus) and $\bar{x}$ (mean of total virus) for each regression line. The broken lines indicate the responses expected for the various dilutions of serum according to relations described in equation 20.

inoculation sites would be expected to contain free virus on the average in a concentration equivalent to pI 8.355, regardless of the amount of antiserum present. The average amount of free virus in those mixtures yielding the 50-percent point, y_{50}, in this series was found to be pI 8.300. This value does not differ significantly from that found in the preceding studies with serum D. R. 848, or from the average value found for untreated purified virus protein, $pI_{50}=8.355$. Each of the remaining statistics in column 1 of table 12 manifests a significant correlation with relative antibody content as measured by serum pD. As in the instance of the similar results with serum D. R. 848 the correlation may be assumed to be linear in each case since any departure from linearity, within the range of virus and serum concentrations covered by this study, is less than can be detected by the method. By using the data of table 12 (A) and following the same mathematical procedures developed above, the following equation was obtained for the relations between free virus, total virus, and dilution of serum D. R. 496.

$$(20)\quad Y=0.4027z^2-0.4355zx+0.1979z+2.7314x-7.1251$$

where Y is the calculated value of y or free virus for any combination of assigned values for x and z.

The expected responses calculated by means of equation 20 are shown by the broken lines of figure 34. Again it was found that the differences between observed and expected response curves were well within the limits of random variation. The results obtained with serum D. R. 496, therefore, confirm the correlations and the type of relation between z, y, and x found for serum D. R. 848. It should be pointed out again that the pD values are purely relative and hold only in the case of a given antiserum. The results with the two antisera cannot, therefore, be compared directly on a basis of equations or charts involving pD values.

DETERMINATION OF THE 50-PERCENT POINT SERUM-VIRUS RELATION FROM RELATIONS ESTABLISHED ON BASIS OF INCUBATION-PERIOD RESPONSES

Still another procedure is available for testing the adequacy of equations 19 and 20 to predict results obtained with sera D. R. 848 and D. R. 496, respectively. The equations developed in the foregoing analyses were derived entirely from incubation-period responses actually measured in test animals. It is possible, however,

from the recorded observations of the frequency distribution of positive and negative inoculation sites to determine the serum-virus mixture in each experiment which gave 50-percent positive and 50-percent negative inoculation sites. These points, designated as x_{50}, have already been given in tables 11 and 12. It should be emphasized that such determinations are of an entirely different character from those of incubation-period measurements, and that the two types of analyses are entirely independent. In a study described in a preceding section a quantitative relation was shown to exist between the percentage positive inoculation sites and the incubation-period response of standard purified virus preparations. It was also demonstrated that the quantity of purified virus required to give the 50-percent point was constant, for all practical purposes, and that this quantity corresponded on the average to $10^{-8.355}$ gm. of virus in 0.1 cc. of inoculum, or $pI_{50}=8.355$. As already pointed out the quantities of free virus, y_{50}, in the 50-percent point serum-virus mixtures were not correlated with serum pD in the case of either serum but were grouped around their respective mean values in random fashion. It was further shown that the average values for y_{50} were pI 8.368 and pI 8.300 for sera D. R. 848 and 496, respectively. These values do not differ significantly from the previously determined value $pI_{50}=8.355$ for standard virus preparations. In the case of untreated or standard virus the free virus is equal to total virus ($y_{50}=x_{50}$), whereas in the case of serum-virus mixtures the total amount of virus, x_{50}, required to give 50-percent positive and 50-percent negative inoculation sites will vary with the amount of serum in the mixture. The quantity of free virus, y_{50}, in any mixture which yields such a result is constant, within limits of experimental error, regardless of the amounts of total virus and serum in the mixture.

Using the established relation between the 50-percent point and the pI of purified virus, namely, $pI_{50}=8.355$, equations 19 and 20, derived in the present study on the basis of incubation-period responses alone, can be tested to see how accurately they predict x_{50}, or total virus, values of the 50-percent serum-virus mixtures observed in the present experiments. For this purpose we may assume that the amount of free virus, y_{50}, is constant for all mixtures giving the 50-percent point and is equivalent to pI 8.355 of purified virus. Therefore, the value of Y in equations 19 and 20 may be considered constant and equal to pI 8.355. The equations may then be solved for x to predict the total amount of virus in the mixture giving this response for any desired concentration of antiserum, i. e., value of z. As an example equation 19 may be employed to predict total virus, x_{50}, required for each amount of antiserum, z, to give an amount of free virus, Y, equal to pI 8.355. With this assigned value of Y, equation 19 may be written

$$(21)\quad 8.355=0.5361\,z^2-0.6515\,zx+0.5788z+3.4084\,x-9.9312$$

where $x=X_{50}$. The notation X_{50} is used to distinguish calculated or expected quantities of total virus required to produce 50-percent positive inoculation sites from the observed values which are indicated by x_{50}. This equation when solved for x becomes

$$(22)\quad X_{50}=\frac{0.5361\ z^2+0.5788\ z-18.2862}{0.6516\ z-3.4084}.$$

Equation 22 may now be used for the determination of expected amounts of total virus, X_{50}, which would be required to give a 50-percent response when mixed with the various quantities of antiserum,

z, employed in the actual experiments. Assigning values of z and solving for X_{50} in equation 22, a series of values were obtained which are shown graphically by the continuous line of figure 35. The circles of this figure represent observed x_{50} values. The broken line represents the best straight line drawn through the observed points by the method of least squares. It will be observed that the continuous line determined by equation 22 and the broken line determined from the observations themselves are very nearly the same although the former curves slightly at its right extremity. For the range of serum concentrations covered by this study, therefore, the average results determined from the observations themselves and those predicted on the basis of equation 22 and a previous estimate of pI_{50} are practically identical. It will be recalled that in the present experiments with this serum, the average value of y_{50} was pI 8.368. This value is very

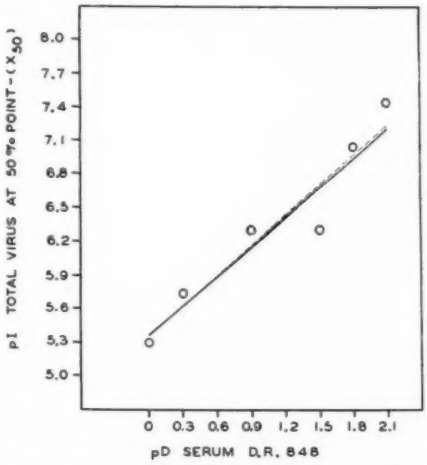


FIGURE 35.—Relation of pI of total virus to pD of serum D. R. 848 giving the 50-percent-point response in experiments of figure 28. Points represent observed relation. The broken line is the best straight line through the points, and the continuous curve indicates the response expected from equation 22.

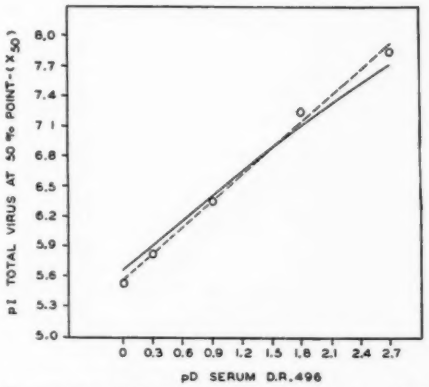


FIGURE 36.—Relation of pI of total virus to pD of serum D. R. 496 giving the 50-percent point response in experiments of figure 34. Points represent observed relation. The broken line is the best straight line through the points, and the continuous curve indicates the response expected from equation 20.

nearly the same as the previously established standard value $pI_{50}=8.355$ used in deriving equation 22.

The results obtained for serum D. R. 496 using the equation

$$(23)\quad X_{50}=\frac{0.4027z^2+0.1979z-15.4801}{0.4355z-2.7314}$$

are shown graphically by the continuous line of figure 36. The circles of this figure represent the independent determinations of x_{50} on the data of the experiments with serum D. R. 496. The broken line represents the best straight line drawn through the observed points by the method of least squares.

It will be noted that the slope of the straight line portion of the continuous line of figure 36 is slightly less than that for the broken line and that the former curves appreciably at its right extremity. Further, the curvature is in the opposite direction and more pronounced than that observed in figure 35 for serum D. R. 848. It will be recalled that the average value of y_{50} observed with this serum was pI 8.300, which is slightly different, but not significantly so

from the standard value, namely, pI 8.355, used in the calculations. Whether the curvature shown by the solid curves of these figures represent trends actually to be expected, or whether they are artifacts due to errors associated with the estimates of the various trends used in the development of the respective equations, cannot be said at the present time. As emphasized above, the few points on which the various trends were determined, and the variability inherent in the method when only small numbers of test animals are employed for the unit observations preclude the detection of curvatures, of the magnitudes here illustrated, by means of the distribution of the observed points themselves. As previously shown, the correlation of x_{50} with pD is highly significant for both sera (tables 11 (B) and 12 (B)), and no departure from a linear relation could be determined from the observed points in either case. However, equations 22 and 23 indicate that the regression of x_{50} on pD actually describes a slight curve in each case as shown in figures 35 and 36, respectively. Determination of their true natures could be accomplished only with extremely large numbers of animals and numerous experiments with a given antiserum since the curvature is less than can be detected under present conditions. Regardless of this fact, however, the equations may be used for predicting x_{50} values within the experimental range when the equation involving a given serum is known, for the observed points fit such calculated values well within the limits of random sampling and fully as well as the observed results fit expected results in the other analyses of this study.

DISCUSSION AND INTERPRETATION

In this paper have been gathered the principal data collected in studies on the purification and properties of the papilloma virus. Investigations of the chemical and physical nature of the material have been few, being limited in general to examinations immediately essential to the development of the ultracentrifugal procedures yielding the purified product. For the most part attention has been directed toward its biological characters chiefly because of the limited amount available and the necessity for adequate criteria for the correlation of results in the various fields. The present work has yielded a wealth of data established for the most part within well-defined quantitative limits. The purified material with which, within the limits of the best methods yet available, are associated the properties of the virus, is peculiarly well suited to quantitative biological investigation and thus extends great promise for the solving of numerous troublesome problems related to the properties of viruses in general and to virus-induced tumors in particular. The results of carefully conducted studies of the purified material are usually clear-cut and to a considerable extent interpretations are self-evident, eliminating the necessity for more than a minimum of speculation leading to further investigation. Physical, chemical, and biological methods of demonstrable adequacy are at hand for extension of quantitative correlation of findings in the different fields.

The purified material subjected to study in the present work has been obtained only from relatively highly infectious extracts of cottontail-rabbit warts. The isolated product is essentially protein in nature and can be obtained in a state of high homogeneity as indicated by examination in the analytical ultracentrifuge. This criterion is employed since it has been the best available for the purpose, and interpretations of the findings have been made with a knowledge of the limits

within which the results have had meaning. The material sediments with the sharp boundary of a homogeneous molecular species and at a rate corresponding to a sedimentation constant of $s_{20°}$=ca. 260×10^{-13} cm. sec.$^{-1}$ dynes^{-1}. In some instances a double boundary is present, the sedimentation constant of which is $s_{20°}$=ca. 375×10^{-13} cm. sec.$^{-1}$ dynes^{-1}. The biological methods now in use have not yet been employed to study the relation of the two components with respect to infectivity or other properties. The conditions responsible for the existence of the two components simultaneously are unknown. The heavier component, however, is always secondary and has not been seen alone.

The amount of protein obtained from infectious extracts is roughly proportional to the infectivity of the initial material. Quantitative studies directed solely toward determining the exact relation have not been made, since the significance of infectivity measurements on crude extracts in relation to physical mass of virus present has not yet been investigated. A general proportionality does exist, however. From cottontail-rabbit wart extracts of very low infectivity a somewhat inhomogenous product may be obtained, and from noninfectious domestic rabbit warts no material remotely resembling the purified product can be obtained. All of the studies thus far made indicate that, within the limits of the methods used, the material is relatively uniform in constitution and biological behavior.

The results of studies on infectivity and complement-fixing capacity of the protein have been described within statistical limits, and application of the data can be made on this basis under the standard conditions described. Aside from the reproducibility of the results, the special advantage of the incubation-period response to the virus should be strongly emphasized. With this relation, the infectivity of any individual inoculum within a broad range can be determined with a relatively high degree of accuracy. In many problems, end point and 50-percent point results are misleading and from them certain quantitative interpretations are difficult to make. This is especially illustrated in the findings of the studies on papilloma-virus neutralization.

The studies on the character of host response to the papilloma-virus protein have yielded findings meriting considerable emphasis. The results indicate that the frequency distribution of positive and negative inoculations with graded amounts of papilloma virus (as well as the corresponding distributions with vaccinia and myxoma viruses) is due chiefly to variations in host susceptibility or response. This is contrary to Parker's concept that the character of the distribution is determined only by virus factors concerned with the chance presence or absence of virus particles in the inocula. Numerous experimental data obtained with both papilloma and vaccinia viruses support the present hypothesis, which attributes to the host the primary role in the determination of the pattern of response to these infectious agents. The concept is further supported by the extensive studies in the field of pharmacology in recent years. Here, the character of host response to many drugs has been attributed in large part to variations in host susceptibility or resistance (*22*, *35*). In the instance of viruses, variations in the susceptibility or resistance of animals to infection are well-known, but the character of this variation has not been extensively studied. Not enough work has been done with viruses from this point of view to make possible more than the suggestion that the character of response to infection with these agents, as in the

instances of many drugs, is determined in the main by variations in host resistance or susceptibility. This is not meant to imply that factors determining host resistance to viruses are the same as those related to resistance to drugs. A demonstrable resemblance between the character of the response to viruses and that to drugs would indicate only that the distributions of variations in the respective factors concerned are the same in the two instances. No information would be furnished relative to the nature of the factors in either case.

The special significance of the present findings in the problem of papilloma-protein infectivity may be briefly stated. If the dose-response frequency is characterized chiefly by host variations, it is clear that Parker's frequency distributions did not correspond to Poisson curves at all but were host-response curves of a character somewhat similar to that of the Poisson curve. It follows, then, that the specific character of the curve *per se* is not a measure of virus infectivity nor does it furnish any information concerning the numerical relation of virus particles and infectious units in the absence of previously determined quantitative relations. Thus, the specific character of the dose-response curve obtained with the papilloma protein has no bearing on the finding that an average of 94,000,000 particles of virus are contained in the 50-percent-point dilution. This observation, then, stands as a direct measure of papilloma-protein infectivity expressed in terms of a definite numerical relation of protein molecules to 50-percent point infectious unit. The same can be said with equal assurance for the results of various investigations with vaccinia.

It should be noted that under certain conditions, as Neymann (*41*) has suggested, variations both in dosage and animal response would be expected to contribute appreciably to the characterization of response. A situation of this sort could exist in instances where a particularly infectious strain of virus is employed and few particles are necessary to infect, as, for example, the strain of vaccinia elementary bodies used by Smadel, Rivers, and Pickels (*29*). If studies were made in animals of wholly uniform resistance or susceptibility, the resultant response curves would be expected to be identical with some distribution described by the Poisson law. Thus, if a group of animals possessed no resistance to the virus, in which instance every intact virus particle would infect, the response curve would be of the form of the Poisson frequency for the distribution of one or more particles. If, however, some uniform degree of resistance were present, some other Poisson frequency should be observed, such as that for the distribution of two or more particles, or three or more, etc. In all of the experiments with the papilloma protein and Parker's results with vaccinia, however, the resemblance between the response observed and the Poisson distribution was always closest to that for one or more particles, and it is clear that such a particle distribution does not exist under the conditions of the experiments with these two viruses.

The most gratifying findings with the purified papilloma protein have been those concerned with the relations between virus and immune serum in the neutralization reaction. Despite variations inherent in the methods used, the results have been readily adaptable to mathematical expression. In addition to the knowledge of the behavior of the papilloma virus, the data provide the basis for a clearer insight into the possible relations involving other viruses. The findings have been presented above and the following interpretations are offered as one view of their meaning.

There is, at the present time, no unequivocal evidence of the nature of that influence of immune serum upon virus which results in the diminution of infectivity of virus with which it is mixed. The phenomenon of "neutralization" is one which is, as yet, detectable and measurable only on the basis of infectivity. Experiments can be set up by mixing immune serum with virus in the test tube under given conditions with respect to virus and serum quantities, temperature, etc., but an idea of what may occur *in vitro* can be gained only by inoculation of the reagents into suitable test tissue. The experiment begun *in vitro* becomes finally one *in vivo*, and with the conversion there are introduced factors concerned with host potentialities. While it is certain that immune serum possesses the capacity to contribute to the lessening of virus infectivity, the mechanism of its action is obscure. It is possible that serum influence is exerted (1) solely on the virus *in vitro* or (2) only on the tissue after inoculation. It is likewise possible (3) that neutralization occurs only as the resultant of the combined and concerted action of immune serum and host influence on the virus, or of host influence on a hypothetical combination of virus and serum which could have arisen *in vitro*. These and other possible interrelations of serum, virus, and test tissues in virus neutralization are generally well recognized. To say that virus is neutralized or inactivated, etc., by immune serum can mean only that the response of the test tissue to a serum-virus mixture is less than that to the same amount of untreated virus. Any term such as neutralization, inactivation, etc., employed to designate the occurrence of the phenomenon at the present, clearly has no meaning with respect to the mechanism involved. However, when virus and immune serum are mixed and inoculated under proper conditions, the net result is one that might be expected if the virus were actually neutralized *in vitro* by the serum. Therefore the diminution in infectivity can be adequately and conveniently discussed through use of the term "neutralization" so long as it is borne in mind that no implications concerning the mechanism are intended.

The purpose of the present experiments has been the study of the relations between virus quantity and antiserum quantity involved in the neutralization of the purified papilloma virus in the presence of anti-papilloma virus-immune serum. It was considered possible in the beginning that, by means of the previously described incubation-period technique which furnished a procedure for accurate assay of virus infectivity, the relations could be found and could be described on a quantitative basis. The experiments, therefore, were made under conditions that were standardized throughout. Further, the studies were limited to a very narrow phase of the general problem. The investigation consisted essentially in the determination of the capacity of a single immune serum to neutralize the papilloma virus and the effects of dilution on this property of the serum. The effects of dilution on the infectivity of the pure papilloma virus with respect to the incubation-period response have already been found, and the various virus preparations employed thus may be considered standard in the exhibition of standard behavior. By mixing successive dilutions of serum with known physical and biological amounts of virus, the absolute neutralizing effect of each dilution of the particular serum could be established. A mathematical relation was derived which described the experimental results well within the limits of accuracy of the methods used. It thus follows that the effect of every quantity of this serum on

every quantity of the virus lying within the experimentally established limits can be determined.

The results of the experiments are clearly described in the mathematical relations derived from them. Their consistency warrants advancement of the empirically established relation as a working hypothesis applicable to the description of papilloma virus-immune serum interrelations in the phenomenon of neutralization. This can be stated mathematically in its general application as

$$(24)\quad y=Az^2-Bzx+Dz+Ex-F.$$

This equation shows the interrelation between (1) the logarithm (of the reciprocal) of serum dilution, z; (2) the logarithm (of the reciprocal) of total virus present, x; and (3) the logarithm (of the reciprocal) of the quantity of free or unneutralized virus, y. Under the standard conditions employed in the present experiments, the coefficients A, B, D, E, and F are constants for a given serum. The general equation has been found to hold for two different sera. For each, however, the respective coefficients were slightly different in value. The significant of the differences, that is, their dependence on the characteristics of various sera, of animal response, or other factors is not known at present. For each antiserum the constants can be determined empirically under established experimental conditions.

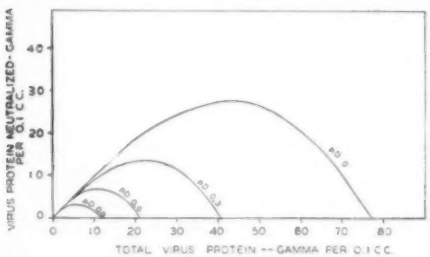


FIGURE 37.—Relation of virus neutralized to total virus mixed with the lower dilutions (pD) of serum D. R. 495.

The interrelations between the three variables described are more clearly visualized if the general equation is broken down into its component parts by showing the relation between only two variables when the third is kept constant.

(1) When a constant volume of a given dilution of immune serum is added to a constant volume of successive dilutions of virus, that is, when z is constant, $z=z_0$, the relation between x and y is

$$(25)\quad y=(E-Bz_0)x+(Az_0^2+Dz_0-F).$$

This is an equation for a straight line indicating that, within the biological range studied under the prescribed conditions, the relation between the logarithm of total virus, x, and the logarithm of free virus, y, is linear (fig.28). It should be emphasized that the equation is limited at the point where free virus is equivalent to total virus or where $y=x$. By subtracting the antilogarithm of the values of free virus calculated from this equation from the antilogarithm of the total virus in the respective serum-virus mixtures, the corresponding values of the amounts of virus neutralized can be obtained. The results thus obtained are illustrated in figure 37 which shows the relations, calculated in gamma of virus per 0.1 cc., between virus neutralized and total virus for several dilutions of immune serum. It is clearly seen that a given quantity of serum does not neutralize a fixed quantity of virus but that the quantity neutralized is dependent on the relative proportions of virus and serum present. For a given quantity of serum, the capacity to neutralize increases with increase in total virus added up to a certain point. At this point, the absolute quantity of virus neutralized reaches a maximum; beyond this point, the capacity to neutralize decreases with increase in the total virus present until a level of total virus is reached

where no effect is seen. It is thus apparent that the neutralization effect observed is definite and quantitative and dependent on the relative concentrations of virus and serum. It is further evident that the proportion of virus neutralized by a given quantity of antiserum diminishes progressively with increase in total virus. Within the limits of the present experiments, the essential relation was not altered by dilution of the antiserum as may be seen in the similar characters of the curves for the respective serum dilutions (fig. 37).

(2) When successive dilutions of a given immune serum are added to a constant quantity of virus, that is, when the total virus, x, is constant, $x=x_0$, the relation between z and y is

$$(26) \qquad y=Az^2+(D-Bx_0)z+(Ex_0-F).$$

This equation represents that of a parabola, and illustrations of the curves obtained under the prescribed conditions are shown in figure 31 in which the logarithm of free virus is compared with the logarithm of serum dilution. It should be emphasized that the equation is limited at the point where free virus is equivalent to total virus or where $x=y$. It is seen that as smaller and smaller quantities of serum were added to a fixed quantity of virus, the relative capacity of the serum to neutralize appears to increase, at first slightly and then more rapidly, in the region of low serum concentration. This increase in relative neutralizing capacity is, of course, a function of the systematic changes in the proportions of serum and virus present, corollary to the relations described in the preceding paragraph. The exact relations giving rise to this observed result may be visualized from figure 38, which is an enlarged section of figure 37. In a hypothetical experiment, for instance, successive dilutions of antiserum may be added to a constant amount, say, to 1.0 gamma of total virus. A line

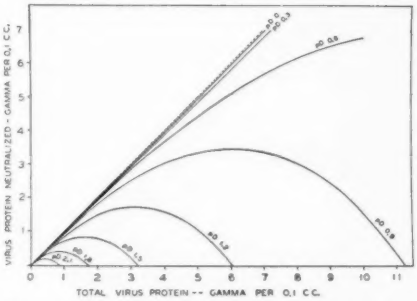


FIGURE 38.—Relation of virus neutralized to total virus mixed with the higher dilutions (pD) of serum D. R. 496.

passed through the point on the abscissa representing this virus quantity and parallel to the ordinate cuts the various serum-dilution curves describing the absolute neutralizing effect of these various quantities of serum on that particular amount of virus. It is obvious that this line does not pass through the same relative points on the various curves.

For the lower dilutions of serum (lower pD values) the line passes through the relatively extreme portion of the ascending left limb of the curve far below the level of the maximum effect which could be exerted by that particular quantity of serum with the proper concentration of virus. For the successive dilutions of serum, however, the line cuts the curves nearer and nearer the peak representing the maximum effect of the various dilutions of serum. Essentially, then, the points on the respective curves are ascending the left limb toward the maximum level, as the serum is progressively diluted. Thus, until the optimum serum dilution is reached, the relative neutralizing power of the serum has appeared to increase. The point is finally reached where, for a given dilution of serum, the line may cut the descending right limb of the corresponding curve beyond the maximum point or miss the curve entirely. Here, the relative concentration

of virus is so great that proportionally less or no effect of the serum is observed. However, in this region the decrease in serum effect is so precipitate within such an extremely narrow experimental range that it would be difficult to demonstrate experimentally because of the limits of the methods employed. Practically, the actual observed results appear to show a progressive increase in serum efficiency with dilution and then a sudden disappearance of effect altogether. This is shown by the junction of experimental curves with the horizontal control lines of figure 31. This paradox is explainable, theoretically, for it is obvious that the observed effects of the serum are determined by the factors discussed above.

(3) When the amount of free virus, y, is constant, $y=y_0$, the relations between z and x are described by

$$(27)\quad Az^2-Bzx+Dz+Ex-(F+y_0)=0,$$

which is the equation of a hyperbola. To be predicted from this equation are those combinations of total virus, x, and serum, z, required to yield always a constant quantity of free virus. One such quantity of free virus which might be chosen for illustration and interpretation is that which would be expected to give 50-percent positive and 50-percent negative inoculation sites. This quantity of virus, having been previously determined, may be substituted for y_0 in the above equation and the value of x for any given value of z (or vice versa) then calculated. The relations under the prescribed conditions are shown in figures 35 and 36 and discussed at length under the heading Neutralization of Purified Papilloma Virus with Immune Serum. Each point in these figures represents the 50-percent-point response for an individual experiment in which a given concentration of serum was mixed with successive dilutions of virus. The experimental points of these figures are grouped closely about the continuous line curves representing the expected response as calculated from the above equation. It should be noted that the experimental points were obtained from relations concerned with the frequency distribution of positive and negative inoculation sites involving data entirely different from those obtained with the incubation-period technique. Thus, the results to be obtained by the former method of analysis are closely predicted by relations determined entirely with the latter. The results may be interpreted as showing that definite relations exist between the logarithm of the total quantity of virus present and the logarithm of serum dilution which will yield the logarithm of quantity of free virus necessary to give the 50-percent point. Further interpretations of these findings are discussed in a subsequent paragraph.

(4) In an experiment in which none of the three variables is constant, the observed results are described by the equation as a whole. Such an experiment is made by mixing a known quantity of virus with a measured quantity of antiserum and titrating successive dilutions of the mixture. An example of the results obtained is shown in figure 33. Of primary significance here is the fact that dilution of the serum-virus mixture results in the progressive increase in the relative amount of free virus. The experimental curve eventually coincides with the control curve showing that at and above the corresponding serum dilution, the remaining increment of serum exerts no effect, and the free virus is equivalent to the total virus. This clearly shows that whatever occurs between virus and serum *in vitro* at a given level of respective concentrations is quantitatively reversible by dilution. In these results are demonstrated for the papilloma virus the quantitative relations existing in the well-

known dilution phenomenon described for other viruses (*45*). Here, again, an apparent change in serum effectiveness on virus is evident. The change, however, appears to be a decrease in serum action with dilution, but again it should be pointed out that this apparent effect is an artifact due solely to the disproportionate dilution of virus and serum.

With respect to the reversibility of the neutralization reaction or "union *in vitro* of papilloma virus and its antibody", the interpretations made above are entirely contrary to those recently reported by Friedewald and Kidd (*39*). In a series of experiments with crude extracts and "washed virus", including absorption of immune body by virus, no evidence of reversibility was observed. Comparison of the findings described here with those reported by Friedewald and Kidd reveals no conflict in the experimental results. Their findings are clearly predictable on the basis of the relations described here and can readily be understood from a theoretical consideration of the mathematical expression of the expected relations. The problems involved will be discussed in full in a forthcoming paper.

Interpretation of the findings in the study of the relations of virus and serum yielding a fixed quantity of free virus, discussed in a preceding paragraph, are of especial interest. Application of the incubation-period method to the study of other viruses has not been made, but recent investigations of serum-virus relations have been made using the 50-percent-point procedure. The results obtained here with this type of analysis, therefore, can be compared directly with those for other viruses for general interpretations.

Such studies have been reported by Parker (*46*) for vaccinia virus and by Horsfall (*47*) for the influenza virus. In the instances of both viruses, it was

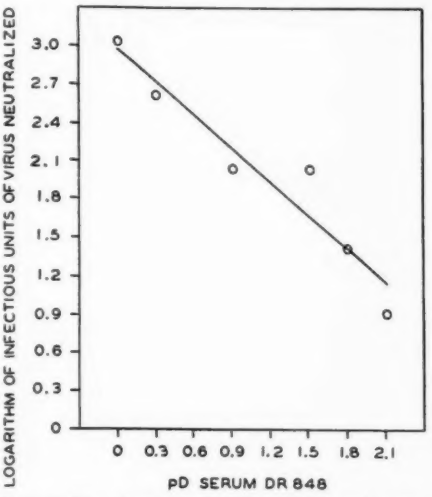


FIGURE 39.—Amount of virus neutralized by the various dilutions of serum D. R. 848 (complement of free virus of fig. 35). Circles represent observed points, and the continuous curve shows the response expected from equation 22.

found, as here, that certain relations exist between the logarithm of the amount of virus neutralized (stated in virus dilution rather than in absolute quantity) and the logarithm of serum dilution. The relation for the papilloma virus is shown in figures 39 and 40 in which the open circles represent experimental points, and the continuous curve the expected response within the limits of the actual experiments calculated from the corresponding equations found in the present study. These figures differ from figures 35 and 36 in that figures 39 and 40 show the relation between serum dilution and virus neutralized rather than between serum dilution and total virus.

The present findings in general provide a basis for the definition of certain limits within which interpretations of data from such 50-percent-point investigations must be confined, and these should be strongly emphasized. In order to obtain the 50-

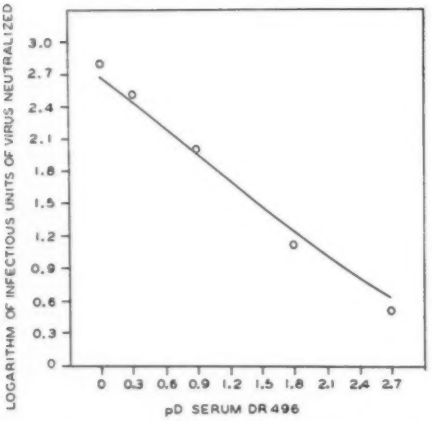


FIGURE 40.—Amount of virus neutralized by the various dilutions of serum D. R. 496 (complement of free virus of fig. 36). Circles represent observed points, and the continuous curve shows the response expected from equation 23.

percent-point response, the primary requisite is a given quantity of free virus. When a given set of virus quantities is mixed with a given series of serum dilutions within the appropriate biological range, a series of 50-percent-points will be obtained. These will show exactly those combinations of serum and virus which yield the requisite quantity of free virus. No information, however, is provided concerning the degree of efficacy of the individual quantity of serum with the particular amount of virus in the mixture giving a 50-percent-point response. It is obvious that if repeated observations are made on a particular serum-virus system, the relations found ought to remain relatively constant and susceptible to general description. This was found to be the case with the papilloma virus here (figs. 39 and 40) and, too, for the influenza virus by Horsfall (*47*) and for the elementary bodies of vaccinia by Parker (*46*). That relations can be shown by means of the 50-percent-point method to exist between serum dilution and virus neutralized appears clear from the work with these three viruses. On the other hand, the limit to which interpretations can be drawn from these data is open to question. This may be illustrated by comparing the interpretations made on the basis of the 50-percent-point method for the influenza virus and the elementary bodies of vaccinia with those that might be made for the papilloma virus under similar conditions. Horsfall concluded that the relation between the logarithm of serum dilution and the logarithm of influenza virus neutralized was linear, that the capacity of a serum to neutralize decreased with dilution, and that, by means of the linear relationship between serum and virus, it was possible to determine a fixed rather than a relative value for the neutralizing capacity of a serum. Parker found that the relation between serum and vaccinia virus was described by a curve of the form of a hyperbola and concluded that serum neutralizing capacity increased with dilution. (It should be pointed out that the latter interpretation could have been made only from the data near the region of the right extremities of the curves of his figures 4 and 5 (*46*). The opposite apparent effect is seen in the left or upper extremities where the action of the serum appears to decrease with dilution.) If interpretations are made for the papilloma virus from 50-percent-point results alone, it could be said that the relation between serum and virus neutralized is linear and that serum effect increases with dilution.

If conclusions must be drawn for the three viruses under discussion here on the basis of the best description of experimental 50-percent-point data alone, profound differences appear to exist in the relations for the respective viruses. On the other hand, there are no *a priori* reasons why the fundamental nature of the neutralization phenomenon should be

different for different viruses. In the present work data have been obtained which were not available in previous investigations. On the basis of the additional information a possible explanation may be advanced for the apparently contradictory findings with the three viruses.

The first point for consideration is whether the logarithmic relationship between serum dilution and virus quantity neutralized is strictly linear or whether it is a curve of one form or another. From his experimental data Horsfall logically concluded that, for the influenza immune serum systems, the relation was linear for both rabbit and ferret immune sera and that both, within the limits of the method, behave similarly. The points obtained by means of the 50-percent-point procedure in the present work with the papilloma virus likewise lie very close to a straight line. In the instances of both viruses, where only 50-percent-point data are considered, interpretations of the nature of the relation can be made only by fitting the points to the best smooth curve through them. It is evident from the data with the influenza virus and those with the papilloma virus that a straight line describes the respective relations about as well as any curve drawn through them. Thus, for practical purposes, the relationship can be considered linear and employed as such as desired. On the other hand, the mathematical relations found for the papilloma virus by means of the incubation-period studies indicate that the relation for the papilloma virus is not linear but hyperbolic. When the experimental points are compared with the curve expected from the equation the points (figs. 39 and 40) actually appear to fit a straight line better than the curve of the predicted hyperbola in one of the curves (fig. 40).

There are several reasons for leaning toward the theoretical curved relationship rather than the apparently acceptable linear findings. In the first place, the mathematical relations established here fit the generality of the observed papilloma-virus data very closely and in the second, the type of curve expected for the papilloma virus from these mathematical relations was demonstrable experimentally by Parker (*46*) in the instance of vaccinia virus. Further it is to be recognized that the investigations with serum virus must all be made within certain limits which, depending on the criteria under study, may be of chemical, physical or biological nature. In the neutralization reaction, the primary criterion is infectivity so that studies on various systems are restricted to the chemical and physical regions limited by this biological criterion to those regions where infectivity can be measured quantitatively within the limits of the method employed. For the papilloma virus, the pI_{50} or the quantity of virus requisite for the 50-percent-point response is 8.355 or $10^{-8.355}$ gm. of virus corresponding to about 94,000,000 virus molecules. With the 50-percent-point procedures, then, only those combinations of serum and virus yielding this quantity of free virus can be studied. In Parker's work with vaccinia, elementary body suspensions were used, and if their infectivity were of approximately the same order as that of the elementary bodies studied by Smadel, Rivers, and Pickels (*29*), the 50-percent-point infections unit would be in the neighborhood of 1 to 10 elementary bodies of mass 8.0×10^{-15} to 8.0×10^{-14} gm. (Parker's thesis that one elementary body always constitutes an infectious unit is of doubtful tenability for reasons previously described.)

The mass-infectivity relations for the influenza virus are not known. Assuming that the serum antibodies for vaccinia and those for the papilloma virus are

reasonably similar with respect to molecular size and general structure, it is obvious that vast differences would exist in the mass and surface, as well as other proportions of virus and serum in the respective systems for the papilloma and vaccinia viruses. Where studies were made with vaccinia in the effective biolcgical range defining the physical limits of ca. 1 to 100,000,000 vaccinal elementary bodies (8.0×10^{-15} to 8.0×10^{-7} gm.) for the papilloma virus the corresponding physical limits were, in the present experiments, from ca. 100,000,000 to 100,000,000,000 molecules ($10^{-8.355}$ to $10^{-5.3}$ gm.). It is conceivable, then, that if the relations for the two viruses could be studied under more comparable physical, chemical, and biological conditions, similar findings might be obtained with both agents and, too, with the influenza virus. If the true relation between logarithm of virus neutralized and logarithm of serum dilution were actually hyperbolic in all these instances, then the region of the general curve included in the results would depend on the limits of the experimental range. Thus, the papilloma and influenza virus results might lie in a region of curvature so slight that the relation could not be detected experimentally from a straight line. For the papilloma data, the extent and direction of curvature is dependent on the values of the coefficients in the general equation. These values were found experimentally, and they contribute to the description of results within the experimental range possible for the papilloma virus. If the biological range could have been shifted, it is possible that coefficients would have been found which would have indicated pronounced curvature within the experimental range. There is some support for this possibility in the present work. In the experiments with serum D. R. 848, the limit of serum dilution was pD 2.1, whereas with serum D. R. 496 the limit was pD 2.7 or a difference of two twofold dilutions. Further, serum D. R. 496 possessed slightly less capacity to neutralize than did D. R. 848. Reference to figure 39 shows that the curve calculated by equation 22 from data of serum D. R. 848 was practically a straight line within the whole range of serum pD (0–2.1) studied. For serum D. R. 496 the curve was comparably linear (fig. 40) between the limits of pD 0 to about pD 1.8–2.1. On the other hand the relation showed definite curvature in the region between pD 1.8 and 2.7. Figure 34 shows that the slopes of the experimental regression lines with the highest dilutions of serum D. R. 496 were relatively low in the region of serum pD 1.8–2.7. In comparison, this region of low slopes was not attained with serum D. R. 848 (fig. 28), where the highest dilution of serum corresponded to pD 2.1.

Another point for question is concerned with conclusions drawn from 50-percent-point data with respect to the neutralizing capacity of a serum or to the effect of dilution on this capacity. It was established in the work with the papilloma virus that the efficiency of a given amount of serum is relative and dependent on the amount of virus present. Therefore, the absolute efficiency of a given serum dilution can be expressed only in terms of its maximum neutralizing capacity in the presence of optimum virus. Thus, comparison of the relative efficiencies of various dilutions of serum can be made only by comparison of their absolute maxima. For instance, from the present data, calculations were made to determine the maximum amount of virus neutralized by the various dilutions of serum D. R. 496. The results are summarized in table 13. It is seen that the relative capacity of this

serum to neutralize was actually decreased with progressive dilution, but it may be noted that the data permitting this conclusion were not available from the results of 50-percent-point studies. Comparison of the quantitative efficiencies of various dilutions of serum can be made at no points on the curves like those of figure 38 other than those of maximum effect, unless mathematical relations are first established, for these curves for various dilutions of sera are not identical in character. For the same reasons, even if the logarithmic relation between serum dilution and virus neutralized were actually linear, when measured by some fixed criterion such as the 50-percent response, it would be possible to obtain a fixed value for the neutralizing *capacity* of a serum only if measurements were made at the maximum points. It would be necessary also to know the exact relations between various sera of different initial strengths, a field not yet investigated.

TABLE 13.—*Maximum quantities of virus neutralized by successive dilutions of serum D. R. 496 in the presence of optimum quantities of total virus*

[Optimum virus concentration and maximum virus neutralized were determined graphically from figures 37 and 38]

Serum pD	Optimum virus concentration (Gamma per 0.1 cc.)	Maximum virus neutralized (Gamma per 0.1 cc.)	Total virus neutralized
			Percent
0	44.9	27.7	61.7
.3	22.5	13.8	61.3
.6	11.5	6.8	59.1
.9	6.0	3.5	58.3
1.2	3.1	1.7	54.8
1.5	1.5	.80	53.3
1.8	.85	.43	50.6
2.1	.44	.198	45.0
2.4	.23	.095	41.3
2.7	.126	.045	35.7

The present results provide a basis for certain limited interpretations with respect to the nature of the serum-virus relations in neutralization. It is evident that within the limits of the experimental conditions, such as the time of exposure of virus to serum, temperature, etc., any combination or relation of serum and virus arising *in vitro* is quantitatively reversible by dilution. Use is made of the term "reversible" with the reservation that, within the limits of the facts, it describes only an apparent effect. However, there is reason to believe that some sort of combination really occurs *in vitro*. Studies of the precipitin reaction (*43*) have shown that combination of antibody with antigen occurs *in vitro*, leading to precipitation of portions of these present. The combination of antigen and antibody constituting the precipitate is reversible by appropriate change in the proportions of the reacting constituents. It should be noted that the relation of serum and virus leading to neutralization behaves similarly under like conditions since the absolute effect of serum is altered not only by dilution but by change in the proportions of serum and virus. For the precipitin reaction, the occurrence of combination *in vitro* is clear. The methods developed for the papilloma virus make possible a direct study of this aspect of the problem, so that it is desirable here only to call attention to the similarity of the results on neutralization (figs. 27, 37, and 38) to those on a different, though probably related, serological system.

The chief point of interest at the present is the fact that an effect which might have been shown by a particular serum-virus mixture can be completely abolished by dilution. This finding makes it quite clear that the actual influence exerted by the serum is brought to bear in its final form leading to neutralization, only after the mixture reaches the test tissue. This interpretation substantiates certain of Sabin's views (*48*) with regard to neutralization of vaccinia virus, namely, that the consummation of the influence of serum in virus neutralization occurs *in vivo* under appropriate conditions. It is obvious that the

final neutralization effect observed is quantitatively related to, and dependent on the proportional concentrations of serum and virus mixed *in vitro*. Therefore, the influence of the host tissue is directly proportional to the same factors. Thus it would appear difficult to evade the concept that host influence is directed toward a definite relation or possible combination of virus and serum determined by the previous association *in vitro*. If the serum effect were directed toward the tissue which, by virtue of "fixed" antibody, acted on the virus, then the effect ought to be related to the proportions of serum and tissue. The opposite, however, is observed, for the significant relation is that of serum and virus established *in vitro*.

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The Peptidase Activities of the Cathepsins of Normal Rat Tissue and of Jensen Rat Sarcoma

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The cathepsins of animal tissues differ from the proteinases of the digestive tract in their pH optima, in their activation by reducing substances, and in their probable function as catalysts in the synthesis of tissue protein. Even before Willstätter and Bamann (*1*) had characterized the proteinases of animal tissue as endocellular enzymes and given them the name, cathepsin, numerous studies on the proteolytic activities of normal and neoplastic tissue had been made. A comprehensive review of the research on the enzymes of tumors which includes this early work has been written by Köhler (*2*).

The cathepsins are of particular interest to the problem of neoplastic growth because of their probable role as catalysts in the synthetic process resulting in the rapid formation of new protein. At present little is known concerning the change in the factors involved in protein metabolism when a normal cell becomes neoplastic, except that the changed factors are self-perpetuating. There are two possible changes which might account for the increased rate of protein synthesis. First, the cathepsin itself may not undergo change, but other metabolic systems which are altered in a malignant tumor, such as the carbohydrate metabolism, may provide a tissue medium which stimulates the more rapid synthesis. Previously published work (*3–6*) from this laboratory has shown that the concentration, degree of oxygenation, and hydrogen-ion concentration are factors which influence the proteolysis or synthesis of proteins in extracts of neoplastic and normal tissues. These same factors appear to influence the rate of nuclear growth in the cell, and Chalkley and Voegtlin (*7*) have found that the rate of cleavage is correlated with the rate of growth of the nucleus. A second possibility lies in definite qualitative changes in the specific activity of the cathepsin which would permit the rapid synthesis of peptides from the same or different amino acids. The results of this type of change in the cathepsin would be the formation of chemically different tumor proteins. As a basis for an enzymatic diagnostic test for cancer, the possibility of a definitely altered cathepsin in cancerous tissue, is therefore, of considerable interest.

As an introduction to a comparative study of the protein metabolism of neoplastic and normal tissues, the cathepsins of Jensen rat sarcoma and of some normal tissues of the rat have been characterized by their peptidase activity on synthetic peptides, glutathione, commercially prepared peptones, and the peptic digests of normal and neoplastic tissue proteins. Unfortunately the normal cell of origin of the Jensen rat sarcoma is not known (*8*). This tumor was used, pending the availability of a malignant tumor with suitable normal control tissue, and it afforded sufficient tissue to establish methods. The question of the effect of a tumor growth

upon other tissues of the host has often been raised. For this reason the cathepsins of the liver, kidney, and spleen of the tumor-bearing animals were studied.

EXPERIMENTAL METHODS

The Enzymes

The tumor and liver catheptic preparations were separated from the globulin fraction of the tumor and liver as described previously (*9*). The spleen and kidney cathepsins were prepared by essentially the same method, except that a more acid reaction was needed to precipitate the globulin fraction of these tissue extracts. It has also been found that more potent enzymes resulted when the tissue extracts and precipitates were maintained in a cold, acid, reducing medium with the addition of M/200 cysteine hydrochloride. Seven types of tissue served as sources for the cathepsins: Jensen rat sarcoma transplanted intramuscularly in the hind legs of Buffalo strain rats, and the liver, spleen, and kidney of normal and of tumor-bearing rats.

The enzymes were tested for catheptic activity on hemoglobin according to a standardized procedure described by Anson (*10–12*). Using this technique a catheptic unit is described as follows: "The initial rate of digestion by 1 unit of cathepsin is such that there is produced per minute at 37° C. in 6 ml. of digestion mixture an amount of color-producing substance not precipitable by trichloracetic acid which gives the same color as 1 milliequivalent of tyrosine" (*10*). The catheptic preparations were activated in varying degrees by cysteine. Since cysteine greatly increased the tyrosine color value produced by the Folin-Ciocalteu phenol reagent (*13*), it was necessary to abolish this added color with formaldehyde. But the formaldehyde also decreased the color produced by the split products of hemoglobin. This decrease in color was ascertained by adding formaldehyde to hemoglobin filtrates of known tyrosine color values. The lower tyrosine color values obtained in the presence of formaldehyde were plotted against the values obtained with the same hemoglobin filtrates without formaldehyde, and by means of the curve obtained corrections were applied to the tyrosine color values obtained by the action of cysteine-activated enzymes. This procedure seemed to be justified since the values obtained with formaldehyde for the different enzymes fell on a straight line when plotted against the known color values in the range of these experiments. Potassium cyanide as an activator also interfered indirectly with this test by reducing the oxidized sulfhydryl groups in the enzymes and substrates, which were not precipitated with trichloracetic acids. With M/100 cysteine hydrochloride as activator, the following activities were obtained for the different catheptic preparations per milligram of protein nitrogen, using 5 cc. of trichloracetic acid filtrate:

Source of cathepsin	$[c.u.]^{Hb}_{mg.\ P.N} \times 10^{3}$
Jensen sarcoma	5.43
Normal livers	5.43
Livers of sarcoma-bearing rats	5.62
Normal kidneys	2.52
Kidneys of sarcoma-bearing rats	3.20
Normal spleens	16.86
Spleens of sarcoma-bearing rats	18.04

While the activities of different catheptic preparations from the same tissue vary, the above values represented the relative activities of the different tissue cathepsins on hemoglobin as determined by this technique. The percentage of protein nitrogen in these cathepsins varied considerably and was inversely proportional to their activity.

The Substrates

The synthetic peptides, glycylglycine, glycylglycylglycine, *dl*-leucylglycine, *d*- and *dl*-leucylglycylglycine, glycyl-*dl*-leucine, *d*-leucine amide, *l*-leucine amide, *dl*-glutamylglycine, carbobenzoxy-*dl*-glutamylglycine, and the natural peptide, glutathione, were used in a concentration of 0.05 mM per cubic centimeter. The *dl*-glutamylglycine and the carbobenzoxy-*dl*-glutamylglycine were prepared according to the method of Grassmann and Schneider (*14*) for the laevorotatory forms. The carbobenzoxy-*dl*-glutamylglycine ethyl ester and the *dl*-glutamylglycine ethyl ester were prepared according to the method described by Bergmann, Zervas, and Fruton (*15*) for the laevorotatory forms. The carbobenzoxy-*dl*-glutamylglycine ethyl ester, because of its low solubility was used in a concentration of 0.2 mM per cubic centimeter. The *dl*-glutamylglycine ethyl ester was used in a concentration of 0.10 mM per cubic centimeter. The *dl*-glutamyl anilide was prepared according to the method given by Hugounenq, Florence, and Couture (*16*), and was used in a concentration of 0.032 mM per cubic centimeter. The α carboxyl group of the glutamic acid is involved in these glutamyl peptides, whereas the glutamic acid in glutathione is combined through its γ carboxyl group. The *d*- and *l*- forms of leucine amide were prepared as described by Yang and Rising (*17*).

The peptic digests of the normal liver and Jensen sarcoma tissue globulins were prepared by adding 0.2 gm. of amorphous pepsin, U. S. P., to 1 liter of a heavy suspension of globulin which had been adjusted to pH 2.0 with hydrochloric acid. After 18 hours digestion at 38 C°., the soluble digestion products were separated by centrifugation, partially neutralized with sodium bicarbonate, and evaporated on the water bath until a concentration of about 1 mg. of amino nitrogen per cubic centimeter was reached. The amino nitrogen in these peptic digests was from 27 to 30 percent of the total nitrogen. Tissue catheptic digests would have been more appropriate to use, but no method was found by which to halt the digestion by the catheptic complex at the peptide stage.

The following commercially prepared peptones were used as substrates in 5-percent concentration: Witte's peptone, which is understood to be a peptic digest of fibrin; Peptone "Roche," prepared by the acid digestion of casein; and silk peptone (Hoffmann-LaRoche) which is prepared from silk fibroin by acid digestion.

The Digestions

The hydrogen-ion concentrations of the digests were maintained with citrate or phosphate buffers after adjustment electrometrically to the optimum pH which had been found for the substrate. The anaerobic digestions were conducted in Thunberg tubes which were evacuated to 5 mm. pressure of Hg. One milligram of dry, lyophilized, catheptic preparation, corresponding to 0.14 mg. total nitrogen was added to each cubic centimeter of digest in all experiments. The digestion temperature was 38° C. Where necessary the enzymes were activated by the addition of M/100 cysteine hydrochloride to the digests. Toluene was added as a preservative.

The hydrolyses of the synthetic peptides was followed by titration of the liberated carboxyl groups in alcohol with an alcoholic solution of potassium hydroxide according to the method described by Grassmann and Heyde (*18*). The increase in amino groups in the highly colored peptone and globulin digests was determined by electrometric formol titrations, using a glass electrode. The principles of this procedure are given by Levy (*19*). The

micro-Van Slyke method was used to determine the extent of cleavage of the peptide bonds in the ester compounds.

RESULTS

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l-Leucine amide was digested most by the normal liver cathepsin and was not attacked by the spleen enzymes. The hydrolysis of this amide by the tumor and kidney enzymes was slight. The unnatural isomer *d*-leucine amide was not hydrolyzed by any of the cathepsins under the same conditions. Assuming that the hydrolysis is asymmetric, the natural isomer of the racemic *dl*-glutamyl anilide was completely hydrolyzed by the normal liver cathepsin, and 80 percent hydrolyzed by the liver cathepsin of the sarcoma-bearing rats.

As shown by table 2, benzoylglycylglycine, or hippurylglycine, was extensively hydrolyzed in 18 hours by the spleen cathepsins. The tumor and kidney cathepsins were inactive toward this substrate. Chloracetyl-*l*-tyrosine was hydro-

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Source of cathepsin	*dl*-Leucylglycylglycine		Glycylglycylglycine		*l*-Leucine amide		*dl*-Glutamyl anilide	
	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
	Hours	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*
Jensen sarcoma	3	7.5	3	0	3	0	3	12
	20	28.5	20	13	20	9	20	16
			72	25	72	7	48	22
Normal rat livers	3	14	3	0	3	10	3	12
	20	32	20	16	20	44	20	12
			72	40	72	46	48	52
Livers from sarcoma-bearing rats	3	25	3	5	3	13	3	6
	20	41	20	30	20	23	20	10
			72	54	72	22	48	40
Normal rat kidneys	3	10	3	3	3	5	3	19
	20	18	20	3	20	14	20	19
			72	13	72	13	48	23
Kidneys from sarcoma-bearing rats	3	14	3	3	3	7	3	19
	20	14.5	20	11	20	9	20	26
			72	38	72	10	48	12
Normal rat spleens	3	42						
	20	59	3	9	3	0	3	19
	48	59	20	39	20	0	20	19
	96	59	72	44	72	0	48	12
Spleens from sarcoma-bearing rats	3	36						
	20	50	3	4	3	0	3	22
	48	50	20	26	20	0	20	15
	96	50	72	51	72	0	48	15

lyzed to a considerable extent by the kidney enzymes, and only slightly hydrolyzed by the liver enzymes. The tumor and spleen cathepsins did not digest chloracetyl-*l*-tyrosine. Glycyl-*l*-tyrosine was digested only slightly by all of the enzymes at pH 5.2.

A comparison of the degree of hydrolysis as determined by the alkalimetric titration and by the Van Slyke amino nitrogen analysis indicates that the ester linkage as well as the peptide bond were hydrolyzed by these enzymes when carbobenzoxy-*dl*-glutamylglycine ethyl ester and *dl*-glutamylglycine ethyl ester were used as substrates (table 3). The high titration values given by the carbobenzoxy-*dl*-glutamylglycine ethyl ester digests under anaerobic conditions with cysteine also indicate that the ester bonds of both optical isomers had been hydrolyzed. The lower titration values given by the *dl*-glutamylglycine ethyl ester digests suggest that the ester bond of only one optical isomer may have been hydrolyzed.[1] Assuming, again, that the peptide splitting was asymmetric, there was no evidence that the unnatural isomers of either of these compounds was hydrolyzed at the peptide bond, since the Van Slyke amino nitrogen determinations did not indicate any significant hydrolysis over 50 percent.

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TABLE 2.—*The hydrolysis of benzoylglycylglycine at pH 4.5, chloracetyl-l-tyrosine at pH 5.0, and glycyl-l-tyrosine at pH 4.5 and pH 5.2 by the cathepsins of different tissues*

Source of cathepsins	Benzoylglycylglycine		Chloracetyl-*l*-tyrosine		Glycyl-*l*-tyrosine			
	Time	Hydrolysis	Time	Hydrolysis	Time	pH	Hydrolysis	
		Cysteine[1]		Cysteine			Cysteine	No cysteine
	Hours	*Percent*	*Hours*	*Percent*	*Hours*		*Percent*	*Percent*
Jensen sarcoma	3	0	3	0	3	5.2	0	0
	20	0	20	0	20		0	0
			48	0	3	4.5	0	
					20		9.5	
					72		39	
Normal rat liver	3	9	3	0	3	5.2	0	0
	20	11	20	16	20		13.5	0
					3	4.5	0	
					20		15	
					72		49	
Livers of sarcoma-bearing rats	3	5	3	0	3	5.2	12	0
	20	28	20	10	20		12	0
					3	4.5	0	
					20		19	
					72		49	
Normal rat kidneys	3	0	3	5	3	5.2	8	9
	20	0	20	47	20		12	12
					3	4.5	0	
					20		12	
					72		29	
Kidneys of sarcoma-bearing rats	3	0	3	7	3	5.2	6	0
	20	0	20	38	20		8	13
					3	4.5	0	
					20		10	
					72		37	
Normal rat spleens	3	59	3	0	3	5.2	0	6
	20	99	20	0	20		0	17
					3	4.5	0	
					20		6	
					72		6	
Spleens of sarcoma-bearing rats	3	31	3	0	3	5.2	0	0
	20	78	20	0	20		0	12
					3	4.5	0	
					20		20	
					72		38	

[1] M/100 cysteine was used to activate the enzymes in these digests.

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The ability of all of the cathepsins to hydrolyze partially the tripeptides, *dl*-leucylglycylglycine and glycylglycylglycine, is shown in table 1. Since the hydrolysis of leucylglycylglycine occurs most readily at the leucine bond, with asymmetric hydrolysis 50-percent digestion by the spleen enzymes may indicate that all of the *l*-leucine had been split off from the peptide which contained the natural optical isomer. In contrast with the extensive hydrolysis of these two tripeptides by the spleen enzymes it is interesting to note the much weaker activity of the kidney enzymes, while the tumor and liver enzymes were equally potent. *d*-Leucylglycylglycine was not digested by any of these enzymes. The dipeptides, *dl*-leucylglycine and glycylglycine, were only slightly attacked by any of the enzymes at this concentration (1 mg. enzyme per cubic centimeter). The dipeptide, glycyl-*dl*-leucine was not hydrolyzed.

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Source of cathepsin	*dl*-Leucylglycyl-glycine		Glycylglycylgly-cine		*l*-Leucine amide		*dl*-Glutamyl anilide	
	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
	Hours	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*
Jensen sarcoma	3	7.5	3	0	3	0	3	12
	20	28.5	20	13	20	9	20	16
			72	25	72	7	48	22
Normal rat livers	3	14	3	0	3	10	3	12
	20	32	20	16	20	44	20	12
			72	40	72	46	48	52
Livers from sarcoma-bearing rats	3	25	3	5	3	13	3	6
	20	41	20	30	20	23	20	10
			72	54	72	22	48	40
Normal rat kidneys	3	10	3	3	3	5	3	19
	20	18	20	3	20	14	20	19
			72	13	72	13	48	23
Kidneys from sarcoma-bearing rats	3	14	3	3	3	7	3	19
	20	14.5	20	11	20	9	20	26
			72	38	72	10	48	12
Normal rat spleens	3	42	3	9	3	0	3	19
	20	59	20	39	20	0	20	19
	48	59	72	44	72	0	48	12
	96	59						
Spleens from sarcoma-bearing rats	3	36	3	4	3	0	3	22
	20	50	20	26	20	0	20	15
	48	50	72	51	72	0	48	15
	96	50						

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Source of cathepsins	Benzoylglycylglycine		Chloracetyl-*l*-tyrosine		Glycyl-*l*-tyrosine			
	Time	Hydrolysis	Time	Hydrolysis	Time	pH	Hydrolysis	
		Cysteine[1]		Cysteine			Cysteine	No cysteine
	Hours	*Percent*	*Hours*	*Percent*	*Hours*		*Percent*	*Percent*
Jensen sarcoma	3	0	3	0	3	5.2	0	0
	20	0	20	0	20		0	0
			48	0	3	4.5	0	
					20		9.5	
					72		39	
Normal rat liver	3	9	3	0	3	5.2	0	0
	20	11	20	16	20		13.5	0
					3	4.5	0	
					20		15	
					72		49	
Livers of sarcoma-bearing rats	3	5	3	0	3	5.2	12	0
	20	28	20	10	20		12	0
					3	4.5	0	
					20		19	
					72		49	
Normal rat kidneys	3	0	3	5	3	5.2	8	9
	20	0	20	47	20		12	12
					3	4.5	0	
					20		12	
					72		29	
Kidneys of sarcoma-bearing rats	3	0	3	7	3	5.2	6	0
	20	0	20	38	20		8	13
					3	4.5	0	
					20		10	
					72		37	
Normal rat spleens	3	59	3	0	3	5.2	0	6
	20	99	20	0	20		0	17
					3	4.5	0	
					20		6	
					72		6	
Spleens of sarcoma-bearing rats	3	31	3	0	3	5.2	0	0
	20	78	20	0	20		0	12
					3	4.5	0	
					20		20	
					72		38	

[1] M/100 cysteine was used to activate the enzymes in these digests.

TABLE 3.—*The hydrolysis of carbobenzoxy-*dl*-glutamylglycine ethyl ester and the* dl*-glutamylglycine ethyl ester by the cathepsin preparations from different tissues*

[The pH of all of these digests was 5.0]

Source of cathepsin	Time	Carbobenzoxy-*dl*-glutamylglycine ethyl ester					*dl*-Glutamylglycine ethyl ester				
		Hydrolysis					Hydrolysis				
		Aerobic			Anaerobic		Aerobic	Anaerobic			
		Cysteine [1]		No cysteine	Cysteine		Cysteine	Cysteine		No cysteine	
		Titration	Van Slyke	Titration	Titration	Van Slyke	Titration	Titration	Van Slyke	Titration	Van Slyke
	Hours	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Jensen sarcoma	3	0		0			0				
	20	98	3	0	135	39	38	57	24	19	16
	48	97	0								
	72				157	43	34	57	21	40	21
Normal rat livers	3			0			28				
	20	104	13	20	156	40	40	42	18	30	16
	72	123	36		166	47	40	71	38	38	20
Livers of sarcoma-bearing rats	3	34		0			22				
	20	95		0	154	53	46				
	72				154	51					
Normal rat kidneys	3	35		0			9				
	20	82		22	113	18	46	31	18	32	20
	72				124	25	57	46	34	38	20
Kidneys of sarcoma-bearing rats	3	10		0			9				
	20	50		13	118	19	43				
	72				155	28	49				
Normal rat spleens	3	31		0			24				
	20	98		0	131	34	47				
	72				150	34	50				
Spleens of sarcoma-bearing rats	3	17		0			19				
	20	37		0	145	53	27	57	23	12	2
	72				157	59	30	75	40	30	11

[1] M/100 cysteine was used to activate the enzymes in these digests.

In order to obtain further information concerning the esterase activity of these catheptic preparations, benzoylglycine ethyl ester was used as a substrate (table 4). All of the enzymes used were markedly activated by cysteine under anaerobic conditions. The high titration value given by the cathepsin from normal rat liver may indicate that this preparation contained an enzyme which was capable of hydrolyzing the benzoylglycine bond. The glycine anhydride formed after removal of the benzoic acid and the ester group would not furnish amino groups for the Van Slyke determination.

The hydrolysis of carbobenzoxy-*dl*-glutamyl glycine as compared with *dl*-glutamylglycine (table 5) shows the effect of masking the free amino group upon the activities of the catheptic enzymes. The slight degree of hydrolysis of *dl*-glutamylglycine was similar to that of the dipeptides, *dl*-leucylglycine and glycylglycine.

TABLE 4.—*The hydrolysis of the benzoylglycine ethyl ester by the different tissue cathepsins*

[All digests were incubated under anaerobic conditions at pH 5.0]

Source of cathepsin	Time	Hydrolysis of benzoylglycine ethyl ester			
		Cysteine		No cysteine	
		Titration	Van Slyke	Titration	Van Slyke
	Hours	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Jensen sarcoma	24	58	0	0	0
	48	81	0	7	0
Normal rat livers	24	109	0	67	0
	48	137	2	87	0
Normal rat kidneys	24	51	0	17	0
	48	59	0	27	0
Spleens of sarcoma-bearing rats	24	59	0	17	0
	48	59	0	14	0

TABLE 5.—*The hydrolysis of reduced and oxidized glutathione at pH 4.5, and carbobenzoxy-*dl*-glutamylglycine and* dl*-glutamylglycine at pH 5.0, by the cathepsins of the different tissues*

Source of cathepsin	Time	SH-glutathione		SS-glutathione	Carbobenzoxy-*dl*-glutamylglycine	*dl*-Glutamylglycine
		Hydrolysis		Hydrolysis	Hydrolysis	Hydrolysis
		Aerobic	Anaerobic	Aerobic cysteine [1]	Aerobic cysteine	Aerobic cysteine
	Hours	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Jensen sarcoma	3				21	0
	20	0	75	21	21	18
	48	0	90	21		
Normal rat livers	3				24	13
	20	21	75	21	24	15
	48	27	97	26		
Livers of sarcoma-bearing rats	3				35	12
	20	38	108	26	34	12
	48	53	115	42		
Normal rat kidneys	3				23	9
	20	8	79	0	25	11
	48	16	79	15		
Kidneys of sarcoma-bearing rats	3				16	4
	20	16	71	10	25	18
	48	16	71	13		
Normal rat spleens	3				32	16
	20	18	129	34	38	11
	48	27	129	48		
Spleens of sarcoma-bearing rats	3				37	15
	20	20	133	37	36	15
	48	20	161	51		

[1] M/100 cysteine was added to activate the enzymes in these digests.

The naturally occurring tripeptide, glutathione, glutamyl-cysteinylglycine, was digested to the greatest extent by all of the cathepsins when its reduced form served simultaneously as substrate and as activator under anaerobic conditions (table 5). There was much less digestion of oxidized glutathione and of reduced glutathione by the enzymes when activated by cysteine under aerobic conditions. The Jensen sarcoma cathepsin, even when used in doubled concentration, did not hydrolyze the reduced glutathione at pH 5.0 under aerobic conditions. No analysis of the digests was made to determine which of the peptide bonds of the glutathione had been hydrolyzed.

The hydrolysis of the peptones prepared

TABLE 6.—*The hydrolysis of peptones by the cathepsins*

[The substrates were dissolved in pH 4.5 citrate buffer containing M/100 cysteine hydrochloride. The degree of hydrolysis was measured by percentage increases in amino nitrogen, NH_2/total N.]

Source of cathepsin	Casein peptone "Roche"		Silk peptone		Witte's peptone			
	Aerobic		Aerobic		Aerobic		Anaerobic	
	3 hrs.	18 hrs.	3 hrs.	18 hrs.	3 hrs.	18 hrs.	3 hrs.	18 hrs.
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Jensen sarcoma tissue	5.6	12.5	3.9	6.2	5.0	12.4	5.2	20.2
Normal rat livers	5.4	11.1	3.0	10.9	4.8	16.9	8.0	18.9
Livers from sarcoma-bearing rats	4.9	12.0	3.9	11.4	8.1	21.2	8.7	31.0
Normal rat kidneys	6.1	13.6	2.8	8.9	5.4	18.9	11.7	23.0
Kidneys from sarcoma-bearing rats	6.3	11.3	2.8	8.5	4.4	16.7	6.6	21.6
Normal rat spleens	6.6	21.4	6.9	14.8	14.6	26.3	15.6	34.9
Spleens from sarcoma-bearing rats	7.7	17.4	6.4	14.5	11.7	22.8	12.5	33.5

by acid digestion of proteins was less than the hydrolysis of Witte's peptone (table 6). Analyses showed that the casein peptone contained 5.9 mg. total N per cubic centimeter and 66 percent unhydrolyzed peptide N; the silk peptone 7.84 mg. total N per cubic centimeter and 81.1 percent peptide N; the Witte's peptone 7.17 mg. total N per cubic centimeter and 79.5 percent peptide N. The extensive digestion of the three peptones by the spleen enzymes was characteristic of these more active proteinases. The slight digestion of the silk peptone by the tumor and kidney enzymes and the almost equal activity of the cathepsins of the normal organs and of the organs of the sarcoma-bearing animals on this substrate are of interest.

Table 7 gives the comparative activities of the different cathepsins on the peptic digests of normal and neoplastic tissue globulins. From these results it appears that the peptic digest of the Jensen sarcoma globulin was hydrolyzed more than the normal liver peptic digest at pH 4.5. Again the normal spleen enzyme was most active. The Jensen sarcoma cathepsin had the same activity as the normal kidney cathepsin. On these substrates the cathepsin of the livers of the rats bearing Jensen sarcoma was more active than that of the normal rat livers. The cathepsin of the kidneys of the sarcoma-bearing rats was definitely weaker than that of the normal kidneys.

TABLE 7.—*A comparison of the activity of the different cathepsins on the peptic digests of normal liver globulin and Jensen sarcoma globulin*

[The hydrolysis was measured in percentage increases in amino nitrogen, NH_2/total N. The substrates contained M/100 cysteine HCl at pH 4.5]

Source of cathepsin	Peptic digest of normal liver globulin		Peptic digest of Jensen sarcoma globulin	
	3 hours	18 hours	3 hours	18 hours
	Percent	*Percent*	*Percent*	*Percent*
Jensen sarcoma	9.0	13.5	6.9	22.6
Normal rat livers	7.8	18.0	5.6	22.9
Livers from sarcoma-bearing rats	13.13	24.0	7.8	28.0
Normal rat kidneys	10.5	13.8	5.6	22.9
Kidneys from sarcoma-bearing rats	6.0	7.8	3.7	16.3
Normal rat spleens	19.5	29.6	18.9	37.8
Spleens from sarcoma-bearing rats	15.3	29.6	16.3	28.3

The hydrolysis of the peptic digests of

TABLE 8.—*The hydrolysis of the peptic digests of Jensen sarcoma globulin and normal liver globulin by Jensen sarcoma and normal liver cathepsins at varying hydrogen-ion concentrations under aerobic and anaerobic conditions*

[The hydrolysis is measured by the percentage increases in amino nitrogen, NH_2/total N]

Digest	Condition and time (in hrs.)	Hydrogen-ion concentration									
		3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
		Pct.	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
Normal liver cathepsin on peptic digest of normal liver globulin	Aerobic:											
	3 hrs.	7.8	12.0	12.0	15.0	16.8	16.8	.....	10.8	.....	3.7	0
	18 hrs.	16.1	23.2	28.1	30.0	29.6	27.7	.....	18.3	.....	3.7	1.5
	Anaerobic:											
	3 hrs.	11.6	12.0	13.5	15.0	20.6	17.6	.....	12.3	.....	5.6	3.7
	18 hrs.	23.6	27.7	37.8	39.0	40.1	39.0	.....	27.0	.....	9.3	4.8
Jensen sarcoma cathepsin on peptic digest of normal liver globulin	Aerobic:											
	3 hrs.	9.0	5.2	7.8	10.1	9.7	8.2	.....	2.2	.....	0	1.5
	18 hrs.	9.0	15.3	24.3	19.1	18.0	19.1	.....	11.6	.....	.37	1.5
	Anaerobic:											
	3 hrs.	6.7	4.5	9.7	13.5	9.0	8.2	.....	6.0	.....	0	.7
	18 hrs.	10.8	22.1	28.5	29.6	31.1	36.7	.....	10.8	.....	0	1.5
Jensen sarcoma cathepsin on peptic digest of Jensen sarcoma globulin	Aerobic:											
	3 hrs.	8.9	10.5	12.0	9.9	11.8	7.6	.....	3.6	.....	2.1	5.5
	18 hrs.	10.5	18.9	20.5	19.9	13.6	14.7	.....	10.5	.....	2.1	4.2
	Anaerobic:											
	3 hrs.	7.1	8.9	12.0	9.2	9.2	11.5	.....	9.2	.....	6.0	4.2
	18 hrs.	12.0	22.8	31.8	27.3	28.9	27.3	.....	16.5	.....	.26	.78
Normal liver cathepsin on peptic digest of Jensen sarcoma globulin	Aerobic:											
	3 hrs.	4.7	6.3	4.9	10.2	8.4	6.5	.....	5.7	.....	.21	.23
	18 hrs.	11.5	13.9	16.0	18.9	20.2	16.0	.....	7.6	.....	0	.05
	Anaerobic:											
	3 hrs.	5.2	6.3	2.8	6.5	9.4	9.7	.....	6.0	.....	.15	.28
	18 hrs.	12.0	19.9	22.6	27.0	33.6	26.5	.....	13.9	.....	3.9	2.63

the normal liver and Jensen sarcoma globulins by the Jensen sarcoma and normal liver cathepsins at varying hydrogen-ion concentrations and under aerobic and anaerobic conditions is shown in table 8. The broadening of the optimal pH range under activated conditions was characteristic of catheptic activity. The limited activity of the enzymes in the alkaline pH range suggests the absence of dipeptidase, amino peptidase, and deamidases in the catheptic preparations. The different pH optima for the same enzyme on the two different substrates might indicate that different specific peptides were attacked. The optimal activity of the tumor cathepsin was slightly less than that of the normal liver cathepsin on both peptic digests.

DISCUSSION

Recently our knowledge of the mode of action of the endocellular proteinases has been advanced by the research of Bergmann, Fruton, and others (*21–23*). The evidence that liver and spleen cathepsins, and the proteinases in general including crystalline pepsin (*24*) and trypsin (*25*) chymotrypsin (*26*) and papain (*27*) can hydrolyze short chain peptides has altered considerably the older conception of the limited activity of proteinases. The crystalline forms of the enzymes, however, may not preclude the possibility that they are complexes of proteinases and peptidases. Because the proteinases digest proteins which contain relatively few free alpha amino and carboxyl groups, Bergmann has defined cathepsins as endoproteinases, signifying that their points of attack are located preferably in the interior of the peptide chain. Further, according to Bergmann, the extent of the splitting of proteins by proteinases is determined by the structure of the protein and by the specificity of the proteinase.

Our experiments showing that the tripeptides are hydrolyzed more than the dipeptides, and also that those showing that carbobenzoxy-*dl*-glutamylglycine ethyl ester and carbobenzoxy-*dl*-glutamylglycine are hydrolyzed to a greater extent than the corresponding peptides in which the amino group is not masked by a carboxyl group, support the postulate that the catheptic enzymes attack more readily the interior of a peptide chain. The marked specificity of the peptidase activity of some of these cathepsins is demonstrated by their extensive hydrolysis of some synthetic peptides and in their inability to attack others.

This specific nature of the attack on the synthetic peptides permits a qualitative and quantitative comparison of the activity of the cathepsins from different tissues and organs, which might throw light upon the function of the organs and the chemistry of their proteins. How much of the specific activity of the cathepsins is related to the metabolic function of the organ and how much is related to the growth of the tissue cells is not known. The fact that the liver catheptic preparations were able to hydrolyze to some extent all of these synthetic peptides might indicate that they are responsible for some of the protein metabolism occurring in the liver. On the other hand, the kidney and spleen cathepsins showed marked specificity for two different acylated peptides. The three peptides, chloracetyl-*l*-tyrosine, benzoylglycylglycine and glycyl-*l*-tyrosine are hydrolyzed extensively in alkaline medium by the carboxypeptidase of spleen and have been regarded as specific substrates for this enzyme (*28, 29*). The evidence that glyclyl-*l*-tyrosine is hydrolyzed by all of the tissue cathepsins, and that the kidney and spleen cathepsins hydrolyze chloracetyl-*l*-tyrosine and benzoylglycylglycine, respectively, shows that these peptides are also hydrolyzed by some catheptic enzymes

at an acid pH. The tumor cathepsin could not hydrolyze chloracetyl-*l*-tyrosine or benzoylglycylglycine.

The relation of the catheptic enzymes to their natural activator, glutathione, as substrate, is of particular interest. Their ability to hydrolyze glutathione was slight under aerobic conditions, but was definitely increased when the glutathione was maintained in the reduced form under anaerobic conditions. The inability of the cathepsin from the Jensen sarcoma to hydrolyze the reduced glutathione aerobically may serve to preserve this important activator for use in the proteolytic and other enzyme systems of the tumor metabolism.

The antipodal specificity of all of these enzymes for the peptides formed from the naturally occurring optical isomers of leucine and glutamic acid is definitely shown. The catheptic enzymes from the tumor and from the normal tissues of the rat were unable to digest the unnatural isomers, *d*-leucine amide and *d*-leucylglycylglycine. With racemic compounds, when the maximum digestion halted at about 50 percent, it has been assumed that a resolution, followed by asymmetric hydrolysis, occurred in which only the natural isomer was attacked. It is of special interest that the cathepsins of Jensen sarcoma tissue did not hydrolyze the *d*-isomerides at all, or the racemic compounds over 50 percent, in view of the evidence presented by Kögl and Erxleben (*30*) that tumor proteins contain considerable quantities of the unnatural *d*-glutamic acid as well as other unnatural amino acids. Since the catheptic enzyme is probably responsible for the synthesis of the tumor protein, it is reasonable to suppose that the tumor cathepsin could hydrolyze a peptide containing the *d*-form of glutamic acid if *d*-glutamic acid were present in tumor protein. One of us (*31*, *32*) has shown that the slight amount of *d*-glutamic acid found in Jensen sarcoma tissue as well as in normal rat liver tissue is an artifact produced during hydrolysis of the tissues with hydrochloric acid.

In support of the evidence presented by Kögl and Erxleben (*30*), Fruton, Irving, and Bergmann (*33*) have reported that extracts of the Bashford mouse carcinoma digested both the *d*- and *l*- forms of leucine amide equally well. The *d*- form was also slightly hydrolyzed by extracts of the Brown-Pearce rabbit carcinoma and mouse sarcoma 180. These authors also found that extracts of the Bashford mouse carcinoma and of a human breast carcinoma digested carbobenzoxy-*d*-glutamyl-*l*-tyrosine. The catheptic enzymes were probably responsible for these hydrolyses since they were activated by cysteine at pH values from 4.6 to 5.2.

No qualitative differences in the activities of the cathepsins from the organs of the normal and of the tumor-bearing rats were observed. The enzymes from the livers of the tumor-bearing rats were more active on most of the synthetic substrates than the enzymes from the livers of normal rats. On the other hand, the cathepsin from the spleens of tumor-bearing rats was less active than the cathepsin from the spleens of normal rats on several substrates. This may possibly be related to the fact that the spleens of the tumor-bearing animals were greatly enlarged and were the only organs which showed any gross changes. This enlargement of the spleen in rats with transplanted tumors has been observed and studied by Okamoto (*34*), Roffo (*35*), and Lambret and Driessens (*36*).

The results with the silk peptone which contains a high percentage of the three amino acids, glycine, alanine, and tyrosine (*37*), are interesting since this substrate offers such slight variation in amino acid combinations that the activities of the cathepsins of the normal organs and those of the tumor-bearing animals were practically the same.

It is reasonable to suppose that the

different enzymes are adapted to the synthesis and hydrolysis of the various amino acid combinations present in the tissues from which they were derived. For this reason the standardization of the activities of all of the endocellular enzymes according to their action on one protein would seem to be without functional significance. The marked hydrolysis of hemoglobin by the normal spleen cathepsin may be correlated with its normal function of red-cell disintegration in the spleen. Although several different types of synthetic peptides have been used in these tests, it is quite probable that the amino acid combinations present in the tissues may not have been represented. Glutathione is a naturally occurring peptide which has been isolated from tissues, but little is known concerning the other peptide groupings of tissue proteins. The peptic digests which were used as substrates contained from 70 to 80 percent of the original peptide linkages of the tissue globulins.

In view of the generally weak activity of the Jensen sarcoma cathepsin on the synthetic peptides and the peptones, it is interesting to note that under optimal conditions of hydrogen-ion concentration and reduced oxygen tension its degree of hydrolysis, 36.7 percent, of the liver peptic digest falls within the range of that of the normal liver cathepsin, 40.1 percent. These results indicate that many peptides are common to both tumor and liver tissue protein. Any qualitative comparison of the peptidase activities of the cancerous and normal tissue cathepsins will depend upon chemical studies of the arrangement of the amino acids in the proteins of tissues supplemented by catheptic activity tests on a great variety of synthetic peptides. The presence of different amino acid combinations in the normal liver and the Jensen sarcoma tissues is suggested by the slightly more acid pH optima in the tumor peptic digests than in the normal liver digest.

SUMMARY

A study has been made of the peptidase activities of catheptic preparations from Jensen sarcoma tissue and from the liver, spleen, and kidney of normal and of Jensen sarcoma-bearing rats. The substrates used have been synthetic peptides, glutathione, commercially prepared peptones, and the peptic digests of Jensen sarcoma and normal liver globulin.

No specific activity was found for the Jensen sarcoma cathepsin among the synthetic peptides used. The kidney cathepsins digested chloracetyl-*l*-tyrosine. The spleen cathepsins hydrolyzed benzoylglycylglycine extensively. Both of these substrates were hydrolyzed by the liver cathepsins.

All of the catheptic preparations were able to hydrolyze oxidized glutathione when activated with cysteine. Their hydrolysis of reduced glutathione under anaerobic conditions was more extensive. The tumor cathepsin did not hydrolyze reduced glutathione under aerobic conditions.

There was no evidence that peptides containing the optically unnatural forms of amino acids were hydrolyzed either by the Jensen sarcoma or the normal catheptic preparations.

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Chemical Studies on the Components of Normal and Neoplastic Tissues

V. The Relative Arginase Activity of Certain Tumors and Normal Control Tissues

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INTRODUCTION

Although numerous studies have been made on the relative enzymatic activity of normal and of tumor tissues (*1*), their value has been difficult to assess. This is due to two causes: (1) The failure to employ comparable normal control tissue for the tumors studied; and (2) the use of an inadequate technique for the enzyme investigations.

Ewing (*2*) has stated that the behavior of a neoplasm is mainly determined by the properties of the tissue of origin. It seems best, therefore, to compare the chemistry of a tumor with that of its tissue of origin (*3*, *4*). Certain exceptions to this procedure are apparent, but studies may be limited to neoplasms of known histogenesis in which the tissue of origin is available in relatively concentrated form. Voegtlin (*3*) has emphasized the care required in the selection of tumors and control tissues and has pointed out that a small amount of unavoidable extraneous material, such as the stroma, may be present. However, necrosis, leukocytic infiltration, and post-mortem degeneration can usually be eliminated. Transplantation of growing neoplastic tissue in animals affords an excellent method for obtaining almost pure material under readily controllable conditions, and we have used this method wherever it has been possible.

In this communication is reported a study of the relative arginase activity of certain pairs of tumor and normal control tissues, as well as that of a number of miscellaneous tumors and normal tissues. This enzyme was chosen for investigation because of the many references in the literature to the greater activity of arginase in tumors as contrasted with normal tissues (*1*). This conclusion was largely based on the relative enzymatic activity of the Jensen rat sarcoma and the Ehrlich mouse carcinoma on the one hand, and normal muscle on the other. As a statement of fact, this may be true. It is doubtful, however, whether it is in any sense a contribution to the problem of cancer. We have found, moreover, that the experimental approach to a study of the relative enzymatic activity of various tissues demands a more extensive technique than that heretofore used. Edlbacher and coworkers (*5*, *6*), and many others (*1*, *7*) made their comparison on the basis of a single determination of the proportion of arginine attacked by the enzyme under wholly arbitrary conditions. This method is obviously meager and insufficient. In order to express the relative enzymatic activity of various tissues in reasonably quantitative terms, we found it necessary to determine the activity over a wide range of varying ratios of enzyme to substrate as well as to conduct studies of the reaction velocity for each tissue. More-

over, the amount of extractable nitrogen from each tissue has had to be taken into consideration.

An exception to the criticism outlined above can be made in the case of the extensive work of Hellerman and coworkers (*8*, *9*) on normal liver arginase. Hellerman and Perkins (*10*) showed that the activity of normal liver arginase follows the course of a monomolecular reaction. We have confirmed this observation and have shown in the present paper that the arginase in each of the tissues studied also follows this order of reaction. Hellerman's finding, therefore, provides the basis of a rational method of comparison of the arginase activity of different tissues.

EXPERIMENTAL PROCEDURE

Material

Although the arginase activity of a wide variety of normal and tumor tissues was investigated, the chief emphasis of these studies was laid on the relative arginase activity of liver, breast, and lymph nodes on the one hand, and of the neoplasms arising from their principal constituents on the other. Tissues were removed from animals immediately after decapitation or ether asphyxiation. They were either extracted at once or were frozen and stored at −10° C. for a few days. There was no evidence that storage in the frozen state has any effect on the arginase activity of the tissues studied.

Gross and Microscopic Descriptions of Tumors

Hepatic tumors of rat

The hepatic tumors were induced by one of the present writers (J. W.) in Osborne-Mendel rats by the oral administration of *p*-dimethylaminoazobenzene (butter yellow).

These tumors were studied pathologically by Dr. Jesse E. Edwards, who submitted the following descriptions:

Tumor 31. The liver bearing the neoplastic tissue was greatly enlarged, weighing 68 gm. Part of the liver was yellow-gray, firm, and nodular and characteristic of cirrhosis. Scattered through the organ and replacing its substance were numerous tumor nodules varying in size from 0.5 to 2.0 cm. in average diameter. Grossly, the tumor tissue varied in character from firm white areas to softer brownish-purple areas. The latter were somewhat cystic.

Tumor tissue was successfully transplanted into the subcutaneous tissues of rats of the Osborne-Mendel strain. The tumor transplants grew rapidly and measured approximately 2.5 cm. in a month. The transplanted tumors were encapsulated, firm, white, and showed a moderate amount of central necrosis.

Microscopically, the original tumors varied in appearance. In some areas they grew as adenocarcinoma with cuboidal cells forming acini lying in closely applied, dense connective tissue. In other fields the tumor cells were arranged in cords and sheets surrounded by a thin fiber of connective tissue which separated the neoplastic cells from endothelial lined blood sinuses.

In the first and second generation transplants the tumor was growing as an adenocarcinoma with a considerable amount of stromal connective tissue. In the third generation there was some tendency toward gland formation on the part of the tumor cells. In other areas the glandlike structures fused with solid cords of cells, and in still others the tumor was growing in the form of closely packed cords with no gland formation. The latter picture was the one encountered almost exclusively in the fourth generation. No fat or glycogen was present in the tumor cells.

In the early generation of transplantation the diagnosis was adenocarcinoma and in the latter hepatoma. This evidence would suggest that the tumor was derived from the parenchymal cells of the liver rather than from cells of the bile ducts.

Tumor 82. The primary tumor arose in the liver of a rat that was deeply jaundiced and showed clay-colored stools. The liver was firm, brown, and mottled with gray areas. The original tumor involved one of the right posterior lobes of the liver. It was firm, white, and measured 2.0 cm. greatest dimension. The mass protruded from the hepatic capsular surface to involve and obstruct the common bile duct in its lower third. Proximally the duct was greatly dilated, measuring

6 mm. in diameter, while distally it was collapsed. The lungs contained six metastatic nodules from 2 to 3 mm. in diameter.

Fragments of the primary tumor were successfully transplanted subcutaneously to rats of the Osborne-Mendel strain.

Microscopically, the primary tumor and the first generation transplant were similar in structure. The cells were fairly large with an abundance of eosinophilic cytoplasm and large vesicular nuclei. The cellular arrangement was in the form of sheets and cords with a slight tendency toward gland formation. No fat or glycogen was present. The diagnosis of this tumor was hepatoma.

The arginase activity of the rat hepatic tumors was compared with that of normal, regenerating, embryonic, and cirrhotic adult rat liver. The remarkable restoration of the liver to its normal size within 2 weeks after partial hepatectomy affords an excellent opportunity for the study of rapidly growing adult tissue (*11*). Residual liver was removed from rats 1, 2, 3, 4, 7, and 10 days after excision of the median and lateral lobes. This provided tissue growing at several different rates in respect to weight, volume, and number of cells. Cirrhotic liver was obtained from rats that had been fed *p*-dimethylaminoazobenzene on a low cystine diet. It appeared histologically as irregular islands of parenchymal cells surrounded by delicate strands of connective tissue. Focal proliferation of bile ducts was noted. The relative arginase activity of adult and fetal rabbit liver (17 days gestation) was also studied.

Hepatic Tumors of Mouse

The tumors in the I and C3H strains of mice were developed by Andervont (*12*). We are indebted to him for this material and to Dr. Hugh Grady for the histopathological findings.

Hepatic tumor No. 1 arose in the liver of a female strain I mouse which had received 11 subcutaneous injections of 2-amino-5-azotoluene. Little variation in its biological behavior or its morphological characteristics has occurred during the 28 passages of this neoplasm in strain I mice. The tumor grows rapidly on subcutaneous transplantation by the trocar method, attaining an average diameter of 2 cm. in 2 weeks when it appears as a circumscribed mass having the color and consistence of liver tissue. Histologically, the neoplasm is composed of atypical polygonal cells resembling those of the hepatic parenchyma. They vary in size and grow in cords separated by sinusoids or occasionally in sheets surrounding a central capillary vessel. Cytoplasm is usually finely granular and the nuclei are round and frequently hyperchromatic. Mitotic figures are numerous and occasional tumor giant cells are seen. There is little stroma except at the periphery of the transplant and little evidence of necrosis or hemorrhage. No bile ducts or typically glandular structures are seen although the cords may occasionally grow about a central space producing a superficial similarity to a gland.

Hepatic tumor A_4 was obtained from Dr. E. W. Emmart and arose in the liver of a C3H mouse which had received 13 subcutaneous injections of 2-amino-5-azotoluene. It has been transplanted through 10 passages in C3H mice. Sixty days after the subcutaneous injection of finely minced tissue, the tumor appears as a firm, yellow-gray, circumscribed mass approximately 2.5 cm. long and 1.5 cm. in width having a small area of central necrosis. This neoplasm reproduces liver architecture closely. Polygonal cells are arranged in sheets or irregular cords separated by sinusoids. The cells closely resemble hepatic parenchymal cells although there is moderate variation in size. Mitotic figures are seen but are not numerous. No bile ducts or evidence of gland formation is noted. The stroma is scanty, and there is little necrosis or hemorrhage.

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Lymphomas

Lymphomas were developed by one of the writers (G. B. M.). Lymphoma Y103 arose spontaneously in a mouse of the dilute brown strain which had enlargement of the liver, spleen, and lymph nodes, and a leukemic blood picture. Subcutaneous transplantation of a piece of affected lymph node into other dilute brown mice was followed in 4 weeks by lesions similar to those found in the original mouse. This neoplasm did not produce a large mass at the site of inoculation. The architecture of the lymph nodes and spleen was obliterated by diffusely growing relatively mature lymphoid cells which varied little in size. Similar cells were found in many of the organs and tissues where they appeared as leukemic infiltrations.

Lymphoma 72942 was similar to Y103 but arose spontaneously in a strain A mouse. Subcutaneous transplantation of neoplastic tissue to other strain A mice was followed in 4 to 6 weeks by the development of lesions similar to those found in lymphoma Y103. The lymphoid cells of lymphoma 72942 were large and relatively immature. They produced a large mass at the site of inoculation.

Lymphoma Y606 arose in the superior mediastinum of a dilute brown mouse that had been painted with a methylcholanthrene solution (*13*). No enlargement of the liver, spleen, or other lymphatic tissues was present at necropsy, although these structures contained tumor cells when examined histologically. Subcutaneous transplantation of a piece of the mediastinal mass produce a large tumor at the site of inoculation, enlargement of regional lymph nodes, and a leukemic blood picture in 3 to 4 weeks. The neoplasm was composed of large lymphoid cells with scanty basophilic cytoplasm and hyperchromatic nuclei that varied slightly in shape. Similar cells infiltrated many organs and tissues.

The observations made on the lymphomas were compared with observations made on normal lymph nodes. These were dissected from the axillary, mesenteric, and inguinal regions of healthy normal adult mice.

Spontaneous mammary tumors

Spontaneous mammary tumors were obtained from female mice of the C3H strain and from hybrid female mice of the F_1 generation that resulted from mating strain C57 Black males to strain A females. Most of the tumors were firm, nodular, gray, solid, circumscribed masses. A few, however, contained cystic areas filled with blood. We are indebted to Dr. Andervont for the tumors from the strain C3H mice (*14*) and Dr. Heston for the tumors from the C57 $\times$ A hybrids.

The arginase activity of the mouse mammary tumors was compared with that of lactating breast and hyperplastic mammary tissue from C3H mice. The hyperplasia was induced by allowing a 6-to-8-mg. pellet of 10 percent stilbestrol in cholesterol to remain in the subcutaneous tissues for 3 weeks. We are indebted to Dr. Shimkin for his advice and assistance in this technique (*15*). The lactating tissue was carefully dissected from mice that had been with their broods at the time of removal. Very little, if any, evidence of milk was observed. For further comparison, the lactating tissue of the rat was also investigated.

The Method for Determination of Relative Arginase Activity

The method used in the present study is similar to that described by many investigators (*5*), namely, incubation of the tissue preparation with *l*-arginine, hydrolysis of the urea formed by urease, and

subsequent estimation of the ammonia formed by the latter reaction. However, since our method of evaluating the *relative* activity is in large measure a new approach, it may be advisable to discuss it in some detail.

For the preparation of the arginase from tissue, we considered it desirable to depart from previous practices of using glycerol extracts, acetone-dried extracts, or pieces of whole tissue. The use of glycerol imposes a change in the reaction medium of unknown extent by considerably depressing the dielectric constant. The acetone-dried extract is composed mostly of denatured proteins, the stability of the dried enzyme decreases with time, and the extract prepared from the dried material is little representative of the tissue from which it is derived. The use of pieces of whole tissue suspended in the reaction mixture during the incubation period with arginine introduces the complication of a separate phase with an unknown rate of liberation of arginase and concomitant substances from the intact tissue into the surrounding medium.

The method which we have adopted is to grind a known weight of the wet tissue with a little quartz sand in a glass mortar. The finely ground material is then suspended in three times its weight of a chilled solution of 0.5M KCl plus 0.03 N $NaHCO_3$ which has a pH of 8.0. The mixture is stirred, a drop of toluene added, and then placed in an ice box at 5° C. for a period of 18 hours. At the end of this time, the mixture is either centrifuged or strained clear through fine gauze. An aliquot of the filtrate is used for total nitrogen determination. This filtrate is used at once for the arginase determinations. Each tissue investigated is treated in the same fashion, and it may be emphasized that determinations are always made on the freshly prepared solution. Although this involves a number of tedious preparations and determinations, the method assures something like a representative extract of the soluble tissue components, some knowledge of the amount of protein and other nitrogenous substances extracted, and the provision of a pH relatively far removed from the optimum of the tissue cathepsin.

The relative arginase determinations for each tissue were conducted in two parts. The first part consisted of a "dilution series," whereby a portion of the extract was progressively diluted with the original chilled extraction-solution of 0.5 M KCl and 0.03 N $NaHCO_3$. The volume of each dilution was maintained at 1.0 cc. Thus the total volume, the salt concentration, and the pH remained constant throughout the series, and the only factor which diminished was the extractable tissue components. We found it convenient to conduct the enzyme reactions in 15-cc. conical centrifuge tubes. To each member in the series 0.4 cc. of a M/15 phosphate buffer at pH 8.0, 0.05 cc. of a 0.2-percent solution of manganese sulfate, and 1.0 cc. of a 0.20-percent solution of arginine monohydrochloride were successively added. For the determination of urea in the tissue, 1.0 cc. of the original, undiluted extracts was treated with 1.0 cc. of distilled water instead of the arginine; phosphate and Mn were added, however, as usual. The arginase reactions were thus carried out at pH 8.0. The mixtures were incubated at 39° for a stated period, then treated with 0.10 cc. of the 2 percent acetic acid and set in a boiling water bath for 10 minutes. The contents of the tubes were cooled, and 20 mg. of powdered jackbean meal (Arlco) was added to each tube. The tubes were shaken vigorously and placed in the incubator for 15 minutes. The urease reactions were thus carried out at pH 6.5. The coagulum in the tubes was then centri-

fuged, the supernatant transferred to aeration tubes, and the coagulum washed with 2 cc. of distilled water. After centrifuging again, the wash water was added to the supernatant in the aeration tubes. The combined supernatant and washings were treated with two drops of caprylic alcohol and 1 cc. of saturated potassium carbonate and aerated in the usual manner. For absorption solution 5.0 cc. of a 0.2 percent sulfuric acid solution was used. After 45 minutes of vigorous aeration, the contents of the absorption solutions were made up to 20.0 cc. with distilled water, 5.0 cc. of Nessler's reagent was added, and the colors developed were compared with those of standard ammonium sulfate solutions. From the amount of total ammonia nitrogen found at each dilution the amounts of ammonia nitrogen derived from the tissue urea were subtracted.

Controls of the jackbean powder and arginine, or of the alkali and arginine were negative. Under the conditions of our experiments, the action of the "argininolytic" enzyme described by Hellerman and Stock (*9*) as being present in jackbean meal was not apparent. Standard solutions of urea were entirely decomposed by the jackbean-meal urease within the time limit given above. A pair of controls were set up for each of the tissues investigated and consisted (1) of tissue extract plus water and (2) of tissue extract plus arginine. Each member of the pair was treated with phosphate buffer and manganese, incubated for 2 hours at 39°, and set in boiling water. No jackbean powder was added to either. The mixtures were immediately aerated after centrifuging and washing. The ammonia found in (1) is that present in the tissue, the ammonia found in (2) is the sum of that found in (1) plus that due to any possible ammoniacal breakdown of arginine during the incubation with the extract. In the case of each tissue, the amount of ammonia found in (1) was practically identical with that found in (2). The amount of ammonia nitrogen found per cubic centimeter of tissue extract ranged from 0 to 0.02 mg.

The second part of the determination of the relative arginase activity consisted in choosing a particular dilution of the original extract which would permit within practicable limits a study of the kinetics of the arginase action. Thus, if the rate of reaction during a 2-hour interval is desired, some care must be exercised in the choice of a particular concentration of extract. As an illustration, undiluted liver extract reaches its maximum effect on arginine within an unmeasurably short period of time. In order to carry out observations of the rate of reaction during a 2-hour period, it is necessary to dilute the orginal extract well over 200 times. When the appropriate concentration has been found, the reaction mixtures are prepared, and the procedures conducted as outlined above. In this connection we have usually studied the rate of reaction over four varying periods of time. The splitting of the arginine by arginase is described in terms of the ammonia nitrogen obtained by aeration, less, of course, by the amount derived from the preformed urea, and the percentage hydrolysis of the arginine is expressed as

$$\frac{\text{Milligrams ammonia N found}}{\text{Milligrams ammonia N for total hydrolysis}} \times 100.$$

The denominator in this equation is 0.266. In order to see whether the action of arginase follows a monomolecular course, the values for the percentage hydrolysis, H, during time intervals, t, are substituted in the formula for the monomolecular reaction:

$$(1)\ K=\frac{2.3}{t}\log\frac{100}{100-H}$$

From the data given in this paper it may be seen that, similar to the findings of Hellerman and Perkins (*10*), the course of arginase activity may be closely described in terms of this order of reaction.

The experimental findings of the arginase activity in the various tissues are described in the following sections. Arginase determinations were performed on several preparations of each tissue. The values obtained were invariably reproducible to within 20 percent. The data on each tissue, usually on one preparation, may, therefore, be considered to be representative.

Effect of Dilution of the Extract on Arginase Activity

The extract of each tissue was progressively diluted as previously described. The total volume, the salt content, the pH, the amount of activator, and the substrate concentration all remained constant—only the extractable components of the tissue diminished in concentration. The various dilutions of each tissue were run simultaneously. The results are given in table 1.[1]

[1] *At very high dilutions, the values marked "0" in table 1 for the amount of ammonia formed after digestion, do not necessarily signify that there is no digestion at these dilutions, but more often that so little ammonia nitrogen is present that our analitical methods are inadequate to determine it with sufficient accuracy.*

Table 1.—*The relation of arginase activity to dilution of the extract*

Time of incubation for arginase 2 hours; the description of the method used is given in the text. The total N is determined on an aliquot of the original strained tissue extract]

Description of tissues	Total N in extract	Total aerated ammonia N after hydrolysis	Aerated ammonia N from urea and ammonia	Hydrolysis of arginine	Q
	Mg. per cc.	*Mg.*	*Mg.*	*Pct.*	
Normal I strain mouse livers.	4.0	0.252	0.045	78	----
	2.0	.254	.023	87	----
	.8	.254	.009	92	----
	.4	.252	.005	93	----
	.2	.252	.002	94	----
	.1	.251	.001	94	----
	.05	.239	----	90	244
	.025	.194	----	73	248
Livers of I strain mice carrying transplanted hepatic tumor.	4.0	.259	.049	79	----
	2.0	.259	.025	88	----
	.8	.260	.010	94	----
	.4	.260	.005	96	----
	.2	.261	.003	97	----
	.1	.260	.002	97	----
	.05	.235	.001	88	238
	.025	.194	----	73	248
Livers of C3H mice carrying transplanted hepatic tumor.	5.25	.299	.094	77	----
	.525	.251	.009	91	----
	.263	.252	.005	93	----
	.131	.252	.002	94	----
	.066	.235	.001	88	----
	.033	.223	----	84	261
	.017	.173	----	65	253
Transplanted hepatic tumor in I strain mice.	4.0	.272	.080	72	45
	2.0	.194	.040	58	46
	.8	.117	.016	38	41
	.4	.077	.008	26	36
	.2	.063	.004	22	38
	.1	.050	.002	18	40
	.05	.033	.001	12	35
	.025	0	----	0	----
Transplanted hepatic tumor in C3H strain mice.	4.65	0.255	0.093	61	36
	.465	.085	.009	28	36
	.232	.064	.005	22	37
	.116	.042	.002	15	30
	.058	.003	.001	12	31
	.029	0	----	0	----
	.015	0	----	0	----
Normal rat liver	4.20	.311	.061	94	----
	2.10	.285	.030	96	----
	.84	.259	.012	93	----
	.42	.256	.006	94	----
	.21	.256	.003	95	----
	.10	.246	.001	92	----
	.05	.231	----	87	235
	.025	.162	----	61	209
	.012	.125	----	47	205
	.006	.098	----	37	203
Liver of rat carrying transplanted hepatic tumor No. 31—third passage.	3.45	.206	.099	74	----
	1.73	.266	.049	81	----
	.69	.266	.019	93	----
	.35	.266	.009	97	----
	.18	.252	.004	93	----
	.09	.204	.002	76	----
	.043	.169	.001	63	180
	.022	.136	----	51	181
	.011	.106	----	40	180
Liver of rat carrying transplanted hepatic tumor No. 31—fourth passage.	4.94	.284	.087	74	----
	2.47	.273	.044	86	----
	.99	.264	.017	93	----
	.50	.271	.008	99	----
	.25	.251	.004	93	----
	.125	.244	.002	91	----
	.062	.203	.001	76	192
	.031	.154	----	58	184
	.015	.111	----	42	170

TABLE 1.—*The relation of arginase activity to dilution of the extract*—Continued

Description of tissues	Total N in extract	Total aerated ammonia N after hydrolysis	Aerated ammonia N from urea and ammonia	Hydrolysis of arginine	Q
	Mg. per cc.	*Mg.*	*Mg.*	*Pct.*	
Liver of rat carrying transplanted hepatic tumor No. 82—first passage.	5.50	0.258	0.088	64	....
	2.75	.265	.044	83	....
	1.10	.265	.018	93	....
	.55	.267	.009	97	....
	.28	.265	.004	98	....
	.14	.265	.002	99	....
	.07	.251	.001	94	....
	.04	.194	.....	73	213
	.02	.146	.....	55	203
Liver of rat fed butter yellow on low cystine diet.	2.47	.272	.046	85	....
	.49	.252	.009	95	....
	.25	.270	.004	100	....
	.125	.231	.002	86	....
	.062	.179	.001	67	169
	.031	.122	.....	46	147
	.015	.096	.....	36	146
Liver of rat fed butter yellow on high cystine diet.	2.47	.267	.052	81	60
	.49	.146	.010	51	64
	.25	.119	.005	43	67
	.125	.066	.002	24	48
	.062	.054	.001	20	51
	.031	.051	.....	19	60
	.015	.035	.....	13	54
Rat hepatic tumor transplant No. 82—first passage.	2.62	.178	.030	48	35
	1.31	.113	.025	33	30
	.52	.087	.010	29	36
	.26	.061	.005	21	32
	.13	.042	.002	15	29
	.065	0	.....	0	....
	.032	0	.....	0	....
	.016	0	.....	0	....
Rat hepatic tumor transplant No. 31—fourth passage.	2.48	.147	.067	30	22
	.49	.058	.013	17	21
	.25	.043	.006	14	21
	.125	.030	.003	10	20
	.062	0	.....	0	....
	.031	0	.....	0	....
Normal female adult rabbit liver.	4.75	.299	.081	82	....
	.48	.279	.008	102	....
	.24	.278	.004	103	....
	.12	.260	.002	97	....
	.06	.246	.001	92	....
	.03	.210	.....	79	254
	.015	.176	.....	66	267
Liver of pregnant rabbit.	3.60	.270	.065	77	....
	.36	.261	.006	96	....
	.18	.261	.003	97	....
	.09	.262	.001	98	....
	.045	.245	.....	92	....
	.022	.189	.....	71	254
	.011	.138	.....	52	233
Rabbit embryo livers 17 days old.	.63	.252	.021	87	101
	.315	.194	.010	69	101
	.157	.146	.005	53	99
	.078	.106	.002	39	92
	.039	.0 9	.001	33	98
	.019	.067	.....	25	92
	.009	0	.....	0	....
Lactating mouse breast.	2.96	.252	.092	60	42
	.59	.143	.018	47	55
	.30	.099	.009	34	51
	.15	.065	.004	23	43
	.075	.047	.002	17	41
	.038	.038	.001	14	42
Hyperplastic mouse breast induced by stilbestrol.	0.95	0.161	0	60	60
	.19	.105	.....	40	69
	.10	.084	.....	31	66
	.05	.072	.....	27	73
	.025	.053	.....	20	68
	.0125	.040	.....	15	64
	.0062	.035	.....	13	71
C3H mouse mammary tumor.	3.00	.306	.059	93	[63]
	1.50	.272	.030	91	[78]
	.60	.225	.012	80	[95]
	.30	.211	.006	77	114
	.15	.168	.003	62	115
	.075	.129	.001	48	114
	.038	.098	.....	37	111
C57xA hybrid mouse mammary tumor.	3.22	.326	.089	89	....
	1.61	.287	.045	91	....
	.64	.257	.018	90	....
	.32	.216	.009	78	113
	.16	.177	.004	65	118
	.08	.140	.002	52	120
	.04	.105	.001	39	114
Normal mouse lymph nodes.	1.96	.145	.076	26	21
	.98	.083	.038	17	19
	.39	.050	.015	13	19
	.20	0	.....	0	....
	.10	0	.....	0	....
Mouse lymphoma Y103.	1.96	.190	.060	49	39
	.98	.107	.030	29	29
	.39	.068	.012	21	29
	.20	.049	.006	16	28
	.10	0	.....	0	....
Mouse lymphoma 72942.	2.00	.117	.021	36	28
	1.00	.079	.010	26	26
	.40	.049	.004	17	24
	.20	0	.....	0	....
	.10	0	.....	0	....
Normal mouse bone marrow.	2.66	.071	.055	6	4
	1.33	.038	.027	4	4
	.53	0	.....	0	....
Mouse lymphoma Y606.	1.80	.305	.084	83	68
	.90	.239	.042	74	75
	.36	.177	.017	60	84
	.18	.134	.009	47	83
	.09	.097	.004	35	77
Normal mouse salivary glands.	4.34	.334	.134	73	46
	.43	.149	.013	51	67
	.22	.112	.006	40	66
	.11	.072	.003	26	55
	.055	.049	.001	18	47
	.028	.040	.....	15	50
	.014	.037	.....	14	59
Normal mouse lung.	4.68	.300	.079	83	49
	2.34	.237	.040	74	55
	.93	.146	.016	49	51
	.46	.109	.008	38	49
	.23	.081	.004	29	47
Normal mouse kidney	1.74	.322	.059	99	[82]
	.35	.097	.012	32	45
	.18	.067	.006	23	41
	.09	.051	.003	18	41
	.045	.041	.001	15	42
	.022	0	.....	0	....
	.011	0	.....	0	....

TABLE 1.—*The relation of arginase activity to dilution of the extract*—Continued

Description of tissues	Total N in extract	Total aerated ammonia N after hydrolysis	Aerated ammonia N from urea and ammonia	Hydrolysis of arginine	Q
	Mg. per cc.	*Mg.*	*Mg.*	*Pct.*	
Normal mouse skin	1.80	0.227	0.110	44	36
	.90	.122	.055	25	26
	.36	.073	.022	19	27
	.18	.046	.011	13	23
	.09	.029	.005	9	21
Normal mouse brains	1.86	.079	.052	10	8
Normal mouse muscle	4.51	.125	.101	9	5
	2.25	.069	.050	7	5
	.90	.028	.020	3	3
Normal mouse spleen	3.36	.142	.113	11	7
	1.68	.080	.056	9	7
	.67	.034	.023	4	5
	.34	0	.....	0	----
	.17	0	.....	0	----
Mouse melanoma	3.50	.273	.105	63	42
	1.75	.206	.052	58	48
	.70	.162	.021	53	58
	.35	.114	.010	39	55
	.18	.072	.005	25	45
	.09	.053	.002	19	47
	.045	.044	.001	16	45
Mouse tumor CR180	1.74	0.226	0.050	66	55
	.87	.161	.025	51	54
	.35	.095	.010	32	46
	.18	.074	.005	26	46
	.09	.053	.002	19	47
	.045	.044	.001	16	45
	.022	0	.....	0	----
Jensen rat sarcoma	1.92	.220	.063	59	47
	.96	.152	.032	45	46
	.38	.111	.013	37	51
	.19	.073	.006	25	44
	.09	.054	.003	19	43
	.045	.041	.001	15	43
Rat lactating breast	5.00	.296	.126	64	[36]
	2.50	.252	.063	71	53
	1.00	.206	.025	68	68
	.50	.158	.012	55	69
	.25	.115	.006	41	65
	.125	.091	.003	33	66
	.062	.068	.001	25	63
	.031	.051	.....	19	61
	.015	.040	.....	15	61

The behavior of the extracts of normal liver and of the mouse mammary tumors on dilution provides a remarkable contrast with that of all the other tissues studied. The arginase activity of liver increases on dilution of the extract until a certain point is reached when it progressively decreases on further dilution. The same phenomenon is observed in the case of the hybrid C57 × A mouse mammary tumor. The enzymatic behavior of the C3H mammary-tumor extract in concentrated solution presents some anomaly, and this will be discussed later. On the other hand, the arginase activity of all the other tissue extracts studied decreases with dilution.

Tentatively, we may attempt to explain this behavior of normal liver extracts on the following grounds: Of all the animal tissues, liver contains by far the greatest proportion of arginase. The latter is presumably either a protein or very similar to a protein (*8*), and as such might be expected to be bound into the closely meshed protein complex of the normal liver cells. When the fresh tissue is extracted, the entire protein complex passes into the extraction solution. The arginase is still concentrated in this complex and cannot free all of its functional groups. On dilution, however, two opposing effects concur. One consists in the progressive dissociation of the protein complex on dilution of the extract, where by more and more of the functional groups of the arginase are liberated and the activity consequently increases. The other consists in a decrease in activity on dilution. Up to a certain dilution the former effect predominates, then the latter influence becomes more effective. The behavior on dilution of normal liver arginase is, therefore, a function of the algebraic sum of the two simultaneous effects. The same explanation may be applicable to the behavior of the spontaneous mouse mammary tumors, which we have shown to have, next to normal liver, the greatest arginase activity of all the tissues studied

(table 3). The dissociation of protein complexes on dilution has often been described (*16*).

The other tissues studied all have an extremely small proportion of arginase in comparison with that of normal liver. On extraction presumably all the functional groups of the relatively small amount of the enzyme are free or nearly free and hence the predominating effect on dilution is simply a drop in activity.

The question that next arises is what is the possible relation of the drop in arginase activity of the tissue extract with dilution to the proportion of total extractable nitrogenous components in solution? Inspection of the values in table 1 shows that while no simple linear relation exists between the two, there is a strong probability of some exponential relation. Calculation of the results reveals that they may be closely described by a cube-root relation, whereby the percentage hydrolysis, H, is roughly proportional to the cube root of the milligrams of total N per cubic centimeter of the extract, TN, as follows:

$$H=\frac{Q}{\sqrt[3]{TN}} \qquad (2)$$

The values of the quotient, Q, are given in table 1, and the near constancy of this empirical relation over the range of concentrations studied demonstrates its validity as a first approximation. The values of Q for the ascending values of activity with dilution of the normal liver and of the hybrid C57×A mammary tumors have been omitted since the relation obviously does not apply. Likewise, although the Q values for the C3H mammary-tumor extracts in the more concentrated solutions are given in table 1, they are enclosed in brackets and are not further considered because of their obviously anomalous character. A physical explanation for this rough cube-root relation is not apparent. It must be emphasized that it has been found to apply under the conditions of our experiments. Whether it would be applicable to a study of arginase under conditions other than those described or whether it would hold under our conditions for other enzymes in tissues must await further experimentation. We have, however, found this empirical relation to be highly useful for present purposes in the comparison of arginase activity in different tissues. The values of Q will be discussed in connection with the work described in the following section.

The Monomolecular Course of the Arginase Reaction

The various tissues were extracted and the extracts adjusted, if necessary, to such concentrations of extractable nitrogen as would yield a gradually increasing amount of enzymatic hydrolysis during a selected time interval. The concentration of extractable nitrogen for each tissue was also chosen within the range where the empirical cube-root relation between concentration and enzymatic activity is valid (equation 2, table 1). The former condition is for convenience, the latter is a necessity for a valid comparison of enzymatic activity in different tissues. The kinetic data are given in table 2. The reaction mixtures were prepared in the same way as previously. It is evident that the course of the enzymatic action with time may be described in terms of equation 1.[2]

[2] *The amount of preformed urea in the tissue extracts studied does not change during the time intervals used.*

TABLE 2.—*The kinetics of arginase in various tissues*

[The values for the percent hydrolysis of arginine at various times, corrected for the urea blanks, are substituted in equation 1 to obtain the values of K]

Tissue	Total N in extract	Period of hydrolysis	Hydrolysis	K
	Mg. per cc.	*Minutes*	*Percent*	$\times 10^3$
Normal I mice livers	0.021	35	50	20.0
		60	71	21.0
		120	83	20.0
Livers of I mice with transplanted hepatic tumor	.060	35	65	31.0
		60	84	31.0
		120	88	29.0
Livers of *C3H* mice with transplanted hepatic tumor.	.021	30	46	20.5
		60	72	20.0
		90	80	17.5
		120	88	17.5
Transplanted hepatic tumor in I mice	1.80	30	11	3.7
		60	23	4.2
		90	27	3.5
		120	41	4.3
Transplanted hepatic tumor in C3H mice	2.33	30	11	3.7
		60	21	3.8
		90	24	3.0
		120	35	3.5
Normal rat liver	.052	30	38	15.9
		60	63	16.3
		90	77	15.9
		120	85	15.6
Liver of rat with transplanted hepatic tumor No. 31—third passage	.023	30	17	6.1
		60	34	6.9
		90	38	5.2
		120	47	5.2
Lactating mouse breast	.296	30	10	3.5
		60	21	3.8
		90	29	3.7
		120	34	3.5
Hyperplastic mouse breast	.05	60	16	2.6
		120	27	2.7
C3H mouse mammary tumor	.10	30	26	10.0
		60	49	11.0
		90	60	10.0
		120	68	9.5
C57×A hybrid mouse mammary tumor	.30	30	35	14.2
		60	57	13.9
		90	72	13.8
		120	81	13.8
Normal mouse lymph nodes	1.96	60	14	2.4
		120	26	2.5
		180	40	2.6
		240	45	2.5
Mouse lymphoma Y103	1.96	30	16	5.9
		60	28	5.4
		90	38	5.4
		120	49	5.5
Mouse lymphoma 72942	2.00	60	19	3.4
		120	36	3.6
		180	50	3.6
		240	58	3.4
Normal mouse bone marrow	2.66	120	6	.55
		180	10	.58
		240	13	.57
Mouse lymphoma Y606	1.80	30	34	13.9
		60	56	13.5
		90	72	13.8
		120	83	14.6
Normal mouse salivary gland	.217	30	12	4.1
		60	22	4.1
		90	30	3.9
		120	40	4.2
Liver of rat with transplanted hepatic tumor No. 31—fourth passage	.019	30	15	5.2
		60	28	5.4
		90	38	5.2
		120	46	5.1
Liver of rat with transplanted hepatic tumor No. 82—first passage	0.036	30	30	11.9
		60	52	12.1
		90	63	10.8
		120	77	12.1
Liver of rat fed butter yellow on low cystine diet	.031	30	14	4.9
		60	27	5.0
		90	34	4.5
		120	42	4.5
Liver of rat fed butter yellow on high cystine diet	.494	30	17	6.1
		60	32	6.3
		90	42	6.0
		120	51	5.9
Rat hepatic tumor transplant No. 82—first passage	2.62	30	14	4.9
		60	28	5.4
		90	40	5.5
		120	48	5.3
Rat hepatic tumor transplant No. 31—fourth passage	2.48	30	8	2.9
		60	15	2.6
		90	25	3.1
		120	30	2.9
Normal female rabbit liver	.019	30	24	9.0
		90	53	8.2
		120	68	9.4
Liver of pregnant rabbit	.014	30	18	6.6
		60	34	6.8
		90	42	6.0
		120	56	6.8
Rabbit embryo livers	.32	30	29	11.0
		60	49	11.1
		90	59	9.7
		120	70	9.9
Normal mouse lung	4.68	30	41	17.6
		60	61	16.0
		90	80	17.5
Normal mouse kidney	.764	30	12	4.4
		90	35	4.7
		120	41	4.3
Normal mouse skin	2.00	30	18	6.6
		60	31	6.2
		90	43	6.1
		120	54	6.4
Normal mouse brain	1.86	60	5	.80
		120	10	.85
		180	15	.88
		240	19	.85
Normal mouse muscle	4.51	60	5	.80
		120	9	.78
		180	12	.74
		240	18	.78
Normal mouse spleen	3.36	60	6	1.0
		120	11	1.0
		180	16	1.0
		240	24	1.1
Mouse melanoma	3.50	60	41	8.9
		120	63	8.7
		180	71	7.0
		240	87	8.5
Mouse tumor CR180	1.74	30	26	10.0
		60	42	9.0
		90	57	9.2
		120	66	8.9
Jensen rat sarcoma	1.92	30	20	7.4
		60	38	7.8
		90	47	6.9
		120	59	7.4
Rat lactating breast	.125	30	10	3.4
		60	21	3.8
		90	25	3.1
		120	33	3.3

The Activity of Regenerating Rat-Liver Tissue

The liver residues were worked up and studied as hitherto. In outward appearance and in the amount of extractable nitrogen these tissues were similar to normal adult liver. No attempt was made to characterize them any further than for the determination of the arginase activity. Since in every case the arginase activity of the regenerating liver tissue was practically identical with that of normal adult liver, it was considered unnecessary to give the experimental results in detail. The final data are given in table 3.

Table 3.—*The relative arginase activity of various tissues*

Tissue	Q	A	q
		$\times 10^3$	$\times 10^2$
Normal I mice livers	246	72	1.1
Livers of I mice with transplanted hepatic tumor	243	76	1.2
Livers of C_3H mice with transplanted hepatic tumor	257	69	1.8
Transplanted hepatic tumors in I mice	40	3	2.0
Transplanted hepatic tumors in C^3H mice	34	2	2.0
Normal rat liver	213	43	1.4
Liver of rat with transplanted hepatic tumor No. 31—third transplant	180	21	2.8
Liver of rat with transplanted hepatic tumor No. 31—fourth transplant	182	19	1.8
Liver of rat with transplanted hepatic tumor No. 82—first passage	208	36	1.6
Liver of rat fed butter yellow on low cystine diet	154	15	1.9
Liver of rat fed butter yellow on high cystine diet	58	8	2.1
Transplanted rat hepatic tumor No. 82—first passage	32	4	1.9
Transplanted rat hepatic tumor No. 31—fourth passage	21	2	2.7
Normal female rabbit liver	260	33	1.7
Liver of pregnant rabbit	244	28	1.8
Rabbit embryo livers	97	15	3.3
Extirpated lobes of normal rat liver	253	45	1.6
Regenerating rat liver 1 days after operation	250	43	1.9
Regenerating rat liver 2 days after operation	251	40	1.9
Regenerating rat liver 3 days after operation	277	43	2.0
Regenerating rat liver 4 days after operation	256	46	1.4
Regenerating rat liver 7 days after operation	254	45	2.2
Regenerating rat liver 10 days after operation	280	47	1.9
Hyperplastic mouse breast	67	7	0
Lactating mouse breast	46	5	3.9
C_3H mouse mammary tumors	114	22	1.1
C_{57}×A mouse mammary tumors	116	21	2.7
Normal mouse lymph nodes	20	2	3.9
Mouse lymphoma Y103	31	4	3.1
Mouse lymphoma 72942	26	3	1.1
Normal mouse bone marrow	4	0.4	2.1
Mouse lymphoma Y606	77	11	5.2
Normal mouse salivary glands	56	7	3.1
Normal mouse lung	50	10	1.7
Normal mouse kidney	42	5	3.3
Normal mouse skin	27	5	6.1
Normal mouse brain	2	.7	2.8
Normal mouse muscle	4	.5	2.2
Normal mouse spleen	6	.7	3.3
Mouse melanoma	49	5	3.0
Mouse tumor C R180	49	8	2.8
Jensen rat sarcoma	46	6	3.3
Rat lactating breast	63	7	2.5

The Relative Arginase Activity

In order to summarize the data in tables 1 and 2 more clearly, the averaged values of Q in the former table for each tissue are collected in table 3. In addition, the averaged values of K for each tissue in table 2 are divided respectively by the cube root of the appropriate values in milligrams per cubic centimeter of the total extractable nitrogen, thus

$$A = \frac{K}{\sqrt[3]{TN}} \tag{3}$$

In addition, it was considered of interest to tabulate the data for the proportion of tissue urea plus ammonia, and for this purpose the results are represented as follows:

$$q = \frac{\text{Milligrams ammonia N from urea and ammonia}}{\text{Milligrams total extractable nitrogen } (TN)} \tag{4}$$

The data for the determination of the values of q are taken from table 1. The values of A and q are given in table 3.

Inspection of the data in table 1 indicates the extent of the misconceptions which may arise in the comparison of enzymatic activity of different tissues.

Let us consider, as an example, the data in this table relating on the one hand to the livers of strain I mice carrying the transplanted hepatic tumor and on the other hand to the transplanted hepatic tumor in strain I mice. The first values in the columns of the dilution series for each tissue are obtained on undiluted extracts prepared in the same way. A comparison of the percent hydrolysis of these extracts reveals 79 and 72 percent for the liver and for the hepatic tumor, respectively. This is the point at which previous investigators have stopped, and under these conditions a comparison of the two tissues might result in the conclusion that the activity of the hepatic tumor arginase was of the same order of magnitude as that of the liver. However, if each extract is progressively diluted while all other factors remain constant, the striking difference in the enzymatic activity of the two tissues is revealed. The same phenomenon is illustrated in the comparison of the activities of the undiluted extracts of normal rabbit liver and rabbit embryonic liver, and even in the case of the other tissues investigated, the differences in activity are insufficiently revealed on the basis of only a single determination of the activity for each tissue. It is obvious that more than identical methods of extraction and single enzymic determinations are necessary for an adequate technique of comparison. The technique which we suggest for the latter purpose may be summarized as follows:

(1) The amount of extractable nitrogen must be known since this value yields information concerning the relative amount of tissue extracted, and the nitrogen value is indispensable for subsequent calculations.

(2) Varying ratios of enzyme to substrate should be employed in view of the data discussed in the previous paragraph.

(3) The possible relation between the extractable nitrogen and the enzyme activity should be determined. Under the conditions of our experiments, an empirical cube-root relation is revealed (equation 2), and on the basis of this relation comparisons between tissues have been here drawn.

(4) It is advisable to determine the order of the reaction rate since the data derived from this study combined with those previously mentioned afford at the present time the most satisfactory quantitative basis of comparison of enzymatic activity in different tissues.

The data in tables 1 and 2 illustrate in part the methods employed in this study. The collected averaged values of Q and A in table 3 reveal the distribution of arginase in various tissues; values of Q and A, as one might expect, approximately parallel each other. The results with the various tissues are summarized in the four following subsections.

Liver and Hepatic Tumors

Embryonic rabbit liver tissue and tumors of the liver in the rat and in the two strains of mice have very much less arginase than has the normal liver of each species. The livers of strain I mice appear to have the same arginase activity as those of C3H mice; similarly, the hepatic tumors of the two strains have approximately the same arginase activity. The presence of the transplanted hepatic tumor in mice of either strain has no apparent effect on the arginase activity of the liver of the tumor-bearing animal. Likewise, the liver arginase of the pregnant rabbit is not affected by the presence of the embryos, and the transplanted hepatic tumor No. 82 in the rat has little or no effect on the liver arginase of the same animal. On the

other hand, the presence of the transplanted fast-growing hepatic tumor No. 31 in the rat apparently causes a marked lowering in the arginase activity of the livers of the tumor-bearing animals. The data for rats carrying the third and the fourth transplants of hepatic tumor No. 31 are consistent in showing this effect.[3]

Rats fed butter yellow together with a low cystine diet develop cirrhosis of the liver. Under the same conditions on a high cystine diet a neoplasm of the liver results. The arginase activity of the cirrhotic liver is lower than normal; the activity of the neoplasm is still lower (table 3). The relatively low value for the liver arginase of the fetal rabbits is also striking.

Regenerating rat liver was found to have the same arginase activity as that of normal adult liver. Like the hepatic tumor, the former is a very rapidly growing tissue, and yet the characteristic physiological property of arginase activity seems to be unaffected by the high rate of growth. For this reason alone, the results on the hepatic tumors are thrown in sharper focus, for in the case of these rapidly growing tumors there is a great drop in arginase activity. The controlled growth of normal and regenerating liver on the one hand and the uncontrolled growth of the hepatic tumor on the other, each described in terms of an enzyme system of widely differing activity, are suggestive of being the result of a physiological environment differing greatly in the two cases. Further investigation is needed, however, on other enzyme systems in these tissues before this point can be settled.

Mammary Tissues

Mammary tissues are in marked contrast with liver and hepatic tumors. Both lactating mammary tissue and hyperplastic mammary tissue induced by stilbestrol are uniformly much lower in arginase activity than are mammary tumors. There is no apparent difference between the arginase activity of the tumor in the two strains of mice studied. Of all the tissues investigated, with the exception of normal liver, the mammary tumors have the greatest arginase activity. Indeed, the values of *A* for the two strains of mammary tumor are of the same order of magnitude as those for the livers of rats carrying transplanted hepatic tumor No. 31 (table 3).[4] This is an unexpected finding, and investigations are in progress to see whether other enzymes are present in such proportions in mammary tumors.

Lymphatic and Blood-forming Tissues

The arginase activity of the normal and tumor tissues here is uniformly low, but the activity of the tumors is relatively greater as compared with the normal control tissues. The differences are not so striking here as they are in the cases of the liver and of the normal mammary and tumor

[3] *The question may be raised as to whether the differences found between liver and hepatic tumors might not be due to a complete extraction of the arginase from the former tissue and to an incomplete extraction from the latter. We therefore re-extracted the treated residues from the first extractions of livers and of hepatic tumors and found consistently that whereas the liver residues yielded a small amount of arginase on the second extraction, the hepatic-tumor residues yielded nothing. The differences, therefore, between these two kinds of tissue are actually somewhat greater than the data in table 3 show.*

[4] *We thought it of interest to see whether milk contained arginase. Raw, fresh cow's milk was investigated, and the results were negative. However, the milk yielded 0.106 mg. of ammonia N per cubic centimeter of milk derived from urea.*

The sera of normal rats and of rats carrying the Jensen sarcoma do not show the presence of arginase. On the other hand, whole rat blood contains a very small amount of arginase derived from the erythrocytes (5). *In terms of the quantity* (Q), *described in table 1, the whole blood gives a value of about 2. The small amount of blood, sometimes present in a few of the tissues studied, contributes almost nothing to the arginase value of the tissues.*

tissues, but reference to the data in table 1 reveals that such differences are real. We may conclude, then, that the normal lymphatic tissues, like the normal mammary tissue, have a lower arginase activity than have the tumors derived from such tissues; on the other hand, the hepatic tumors uniformly have a much lower arginase activity than the normal liver tissue. No generalization, therefore, can cover all the cases studied.

Miscellaneous Normal and Tumor Tissues

Table 3 gives data on a relatively wide variety of tissues. The arginase activity is uniformly low compared with that of normal liver or mammary tumors. The lowest values are found in the case of brain and of spleen. The low value for arginase in the latter organ stands in marked contrast with the very high values for catheptic activity in spleen. It seems likely that the distribution of enzymes may not be the same in various tissues.

The values of q in table 3, which represents the percentage of free ammonia plus urea nitrogen of the total tissue extract nitrogen, have an independent interest of their own. There is no obvious relation between the arginase activity of any one tissue and the amount of free ammonia and urea present in the tissue. The rather high proportion of these components in the embryo livers as compared with normal and with tumor livers is noteworthy. The greatest proportion of these components is found in normal mouse skin. There is no change whatever in the content of preformed urea in any of the tissue extracts during the times of incubation.

Up to this point we have considered only the total extractable nitrogen in the tissue extracts and have based our calculations exclusively on the figures for this quantity. It is possible that another method of calculation, based on the amount of protein nitrogen in the extract, would have been preferable. In fact, we determined the amount of protein nitrogen in the extracts of livers and hepatic tumors, breast and mammary tumors, and the normal and neoplastic lymphatic tissues. The protein nitrogen was derived on the basis of the material precipitated by 5 percent trichloracetic acid. The values of total and of protein nitrogen for the first two kinds of tissues were not very different; the differences were only little greater than the ammonia plus urea nitrogen in the extracts. The differences in total and protein nitrogen were rather larger in the case of each of the third kind of tissues. Substitution of the values of the cube root of the protein nitrogen in equations 2 and 3 gave values of Q very little different from those obtained on the basis of the total nitrogen. This is, of course, due to the fact that the relatively small differences between total and protein nitrogen are rendered even smaller when the cube root of each is taken. However, we rejected the use of the values for the protein nitrogen on the following grounds: These values are in reality a function of the concentration of trichloracetic acid used, and of the relative concentration of protein in solution. Different concentrations of trichloracetic acid will yield different amounts of protein nitrogen. The two hundredfold dilution of an extract of liver will not yield, with the same concentration of trichloracetic acid, one two-hundredth of the original content of protein nitrogen. Changes in the state of protein aggregation with dilution render the values of protein nitrogen rather questionable. On the other hand, values for the total extractable nitrogen are unequivocal and independent of dilution or any other known effect.

The pH Optimum of Arginase Activity in Normal and Tumor Tissues

In every case, the arginase activities in tables 1–3 were determined at pH 8.0. The optimum pH for this enzyme in the normal liver tissue of various species is about 9.6 (*6*). Only one observation on the pH optimum of arginase in a tumor is available in the literature. Edlbacher, Kraus, and Leuthardt (*6*) have reported that the arginase of Ehrlich mouse carcinoma tissue has an optimum activity in air at pH 7. These workers furthermore claim that this optimum is shifted to pH 9.2 under anaerobic conditions. We have investigated the arginase activity as a function of the pH for a number of tumors and normal control tissues in order to see whether the differences which we found might not be due to considerable differences in the pH optima of these tissues.

The determinations were conducted, as they have been throughout, under aerobic conditions.[5] The pH of the various tissue extracts were adjusted with solutions of acetic acid and of sodium hydroxide of varying strength. Before the addition of urease, the reaction mixtures were adjusted to pH 6.5. The determination of the pH was performed with a glass electrode. The data are given in table 4.

The data in table 4 suggest that the optimal pH range for the arginase of the various tissues studied occurs at 9 to 10. The differences shown, for example, by the liver and hepatic tumor tissues at extreme pH values are relatively unimportant and may well be due to the fact that the total protein concentrations were considerably different in the two extracts. We may conclude that there is no significant difference between the arginase activity of the tumors and the normal control tissues as a function of the pH.

[5] *The reaction tubes are always tightly stoppered during the incubation period.*

Table 4.—*Arginase activity as a function of the pH*

[The time of incubation for arginase was 2 hours]

Tissue	Total N in extract	pH	Hydrolysis
	Mg. per cc.		*Percent*
Livers of I mice carrying transplanted hepatic tumor	0.025	3.7	8
		5.4	8
		6.3	62
		7.7	75
		8.7	79
		9.5	98
		10.9	92
		12.1	16
Transplanted hepatic tumors in I mice	2.0	3.9	0
		5.1	29
		6.3	54
		7.5	58
		8.5	69
		9.5	76
		10.3	63
		11.9	0
Lactating mouse breast	.59	4.6	5
		5.8	11
		6.2	20
		7.2	30
		8.0	47
		8.8	48
		9.5	52
		11.2	10
Mammary tumor in C57 × A mice	.140	4.3	0
		5.8	8
		6.4	35
		7.4	52
		8.3	70
		9.2	92
		10.2	66
		11.9	0
Normal mouse lymph nodes	1.96	4.0	0
		5.0	4
		6.0	7
		7.1	8
		8.0	23
		9.1	40
		10.2	13
		11.5	3
Mouse lymphoma Y103	1.96	4.1	0
		5.2	4
		6.0	24
		7.2	40
		8.1	52
		9.1	65
		10.0	33
		11.2	2
Mouse lymphoma 72942	2.0	4.0	0
		5.0	0
		6.2	18
		7.4	34
		8.3	37
		9.1	71
		10.1	59
		11.0	30

Arginase Activity Without Added Activator

In the study of the effect of varying the amount of extractable nitrogen while all the other factors remained constant (table 1), one must consider not only the changing ratio of substrate to extractable nitrogen but also the changing ratio of

added manganese activator to the latter quantity. Is the possible effect of the added activator a contributing factor to the differences in arginase activity found between tumors and the normal control tissues? We investigated normal rat liver and the transplanted rat hepatic tumor No. 31 under closely comparable conditions with and without added manganese. Moreover, we dialyzed separately extracts of the livers of I mice and of the transplanted hepatic tumors in I mice. The dialysis was conducted for 3 weeks in the cold room at 5° C. against frequently changed distilled water. At the end of this period, the activity of the respective dialysates were investigated in the presence and in the absence of added manganese. The time of incubation in each case was 2 hours. The results are given in table 5.

TABLE 5.—*The effect of added manganese on the arginase activity of tissue extracts*

Tissue	Total N in extract	Hydrolysis	
		0.05 cc. water added per cc. of extract	0.05 cc. of 0.2 percent Mn sulfate [1] added per cc. of extract
	Mg. per cc.	*Percent*	*Percent*
Normal rat liver	2.10	85	84
	.84	96	97
	.42	98	101
	.21	95	95
	.10	89	92
	.05	76	77
	.025	48	55
	.012	36	40
Transplanted hepatic tumor No. 31—fourth passage.	2.48	26	31
	.49	20	20
	.25	16	17
	.125	10	12
	.062	0	0
	.031	0	0
Dialysate of extract of strain I mice livers.	1.04	11	19
	.52	9	18
	.26	8	13
Dialysate of extract of transplanted hepatic tumor of strain I mice.	.68	0	0
	.34	0	0
	.17	0	0

[1] 0.1 percent Mn sulfate used for the dialysates.

It is evident that the addition of manganese sulfate has a very slight activating effect upon the normal rat liver arginase in dilute solutions of the extract and upon the arginase of the hepatic tumor. It is quite likely that the extract of each tissue has orginally an adequate amount of natural activator derived from the tissue so that addition of manganese produces a relatively small effect. In any event, it is significant in the case of normal liver extract that the increase of arginase activity with dilution followed by a decrease on further dilution occurs in the absence of added manganese.

During the dialysis of the mouse liver and hepatic tumor extracts, not only were the natural concomitants of the arginase presumably removed (*17*), but the extracts also underwent extensive autolysis. The values for the liver arginase in the dialysate studied were quite low (table 5). On treatment with manganese the activity was increased. The arginase of the transplanted mouse hepatic tumor was apparently completely destroyed during the dialysis procedure.

dl-HOMOARGININE AS A SUBSTRATE FOR ARGINASE

The range of substrates containing the guanidine group which are attacked by arginase has been discussed by Hellerman and coworkers (*8*, *9*). It has been reported by one of us that racemic homoarginine (*dl*-epsilon guanido alpha amino-n-caproic acid) was not observably hydrolyzed by normal liver arginase (*18*). The period of incubation was 30 minutes. Some of the original preparation of homoarginine sulfate was available, and it was considered to be of interest to reinvestigate its use as a substrate for the arginase of normal and of tumor tissues, using considerably longer periods of incubation. Any considerable differences between the arginase of the two types of tissues might be expected to be reflected in the behavior of this substance as a substrate. The reaction mixtures were prepared in the

same way as before; the homoarginine sulfate solution was 0.40 percent. One cubic centimeter of the solution of substrate was employed in the tests, and on the basis of a complete asymmetric hydrolysis 0.224 mg. of ammonia nitrogen would be obtained. In order to see whether any part of the ammonia nitrogen found after hydrolysis was derived from the possible action of *d*-amino acid oxidase on the substrate, the usual blanks on (1) tissue extract plus water and (2) tissue extract plus homoarginine were performed. The values found for (1) and (2) were again practically identical. The results of the action of the tissue extracts on homoarginine are given in table 6. For comparison, the hydrolysis of arginine by the tissues under the same conditions is also given in the table.

TABLE 6.—dl-*Homoarginine as a substrate for arginase*

[Time of incubation was 4 hours. Calculations are based on asymmetric hydrolysis of homoarginine]

Tissue	Total N in extract	Hydrolysis of homoarginine	Hydrolysis of arginine
	Mg. N per cc.	*Percent*	*Percent*
Livers of I mice carrying transplanted hepatic tumors.	3.70	9	100
Transplanted hepatic tumors of I mice.	3.15	1	90
Livers of C3H mice carrying transplanted hepatic tumor.	5.25	9	90
Transplanted hepatic tumors of C3H mice.	4.65	0	86
Normal rat liver	4.20	12	98
Liver of rat carrying transplanted hepatic tumor No. 82—first passage	5.50	27	89
Transplanted rat hepatic tumor No. 82—first passage	2.62	0	86
Liver of rat carrying transplanted hepatic tumor No. 31—third passage	3.45	15	77
Transplanted rat hepatic tumor No. 31—third passage	3.14	0	53
Lactating mouse breast	2.96	0	77
Mouse mammary tumor C57×A	3.22	0	83
Normal mouse lymph nodes	1.98	0	45
Mouse lymphoma Y103	3.36	0	84

The homoarginine is attacked at a very much slower rate by arginase than is arginine. The amount of homoarginine hydrolyzed is proportional to the arginase activity of the tissue. Thus, liver tissue hydrolyzes homoarginine to some extent, whereas all the other tissues studied show no effect under the conditions employed. The experiments with homoarginine are, therefore, a rough confirmation of the results for the distribution of arginase for various tissues when arginine was used as substrate (tables 1 and 2).

SUMMARY AND CONCLUSIONS

1. The activity of arginase in the tumors of three different kinds of tissues as compared with their respective normal controls was estimated. The tumors included (1) transplanted hepatic tumors of rats and two strains of mice, (2) spontaneous mammary tumors of two strains of mice, and (3) lymphomas of mice. For normal controls there were used, respectively, regenerating livers and the livers of the tumor-bearing and normal animals, lactating and hyperplastic mammary tissue induced by stilbestrol, and lymph nodes and bone marrow. The arginase activity of rabbit embryonic liver was also compared with that of the livers of pregnant and nonpregnant female rabbits. In addition, the arginase activity was determined of a wide variety of normal and miscellaneous tumor tissues for which no normal control tissues are known.

2. The relative activity of each tissue was estimated (1) by varying the amount of extractable nitrogen in the extract solutions used while the substrate concentration and all other factors remained constant, and (2) by studying the reaction velocity of the enzyme at one value of the extractable nitrogen. The latter study revealed that the arginase in all of the tissues studied follows a monomolecular reaction. The comparison of the enzymatic activity was made possible by the discovery of an empirical relation between

the activity as expressed by percent hydrolysis of arginine and the cube root of the value for the total nitrogen per cubic centimeter of extract solution. This relation was found to describe the data obtained over the entire range of concentrations studied for all of the tissues save normal liver and the spontaneous mammary tumors. Only in very dilute solutions of the extracts of the latter two kinds of tissues did this relation hold. These had the greatest arginase activity of all the tissues studied. The writers have tentatively interpreted the failure of the exponential relation to hold in concentrated solutions to an aggregation of proteins with saturation of some of the active centers of the enzyme.

3. The relative activity of each tissue was expressed in two ways: (1) By determining the average value of the ratio of percent hydrolysis to the cube root of total extractable nitrogen per cubic centimeter of extract over a series of varying values of the latter; and (2) by determining the monomolecular reaction constant and dividing this constant by the cube root of the total extractable nitrogen per cubic centimeter. Both methods yielded nearly parallel results.

4. The results on the pairs of tumor and normal control tissues indicate that (1) the arginase activity of the transplanted hepatic tumors and of the embryonic liver is very much less than that of normal liver, (2) there is no apparent effect of the transplant on the liver arginase of the same animal except in the case of a particularly rapidly growing hepatic tumor in rats, (3) regenerating and normal adult livers have the same activity, (4) the arginase activity of the spontaneous mammary tumors is very much greater, that of the lymphomas only slightly greater than that of the respective normal control tissues, (5) the activity of the same tissue from different strains is practically identical, (6) the activity of normal lactating and stilbestrol-induced hyperplastic mammary tissues is very nearly the same, (7) the pH optimum of arginase is the same for the tumors and controls, (8) the relative activity of hepatic tumor and liver is not significantly affected by the presence or absence of added manganese, (9) prolonged dialysis, however, of normal liver leads to a preparation which is strongly activated by manganese whereas prolonged dialysis of hepatic tumor extract results in apparently complete loss in enzyme activity, and (10) racemic homoarginine is hydrolyzed to a small extent by normal liver extract, not at all under the same conditions by extracts of hepatic or mammary tumor.

5. Most of the miscellaneous normal and tumor tissues studied yielded relatively low values for arginase activity as compared with that of normal liver and spontaneous mammary tumors, but were of the same order of magnitude as the hepatic tumors and lymphomas. The lowest values were found in bone marrow, brain, and spleen.

6. The values of preformed urea nitrogen in the tissue extracts ranged mostly from 1 to 3 percent of the total extractable nitrogen. The largest value was for that found in the skin. There is no apparent relation between the content of preformed urea in the tissue and the activity or arginase in the tissue.

7. This study was designed as a partial characterization of, and comparison between certain tumors and normal control tissues. Until more data are available concerning other enzyme systems in these tissues, we are reluctant at the present time to discuss the relation of our findings to the physiology of normal and neoplastic tissues.

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Induction Of Tumors In Guinea Pigs With Subcutaneously Injected Methylcholanthrene

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Most experimental cancer research has been confined to mice and rats. The extension of the work to other species, undoubtedly desirable for the wider application of the information, has been limited for several reasons: the difficulty of maintaining larger animals in the laboratory, the lack of genetically homogenous stocks, and the purported resistance of the animals to the induction of experimental tumors.

The National Cancer Institute was fortunate in securing, in 1940, guinea pigs of three highly inbred strains from Dr. Orson N. Eaton, of the Beltsville Experiment Station of the United States Department of Agriculture.[1] The history of the development of these strains (or "families") has been recorded by Wright (*1*), Wright and Eaton (*2*), and McPhee and Eaton (*3*). Practically complete homozygosis was reached in 1915, and the differentiated characteristics have been persistent (*2*). Differentiation has been most obvious in the fixation of such characteristics as coat color and tendency toward polydactylism and toward the production of particular types of monsters (*1*, *2*). There has been also considerable fixation in the size at birth, at weaning, and at maturity, in differential mortality, and in the degree of fertility as manifest by the size and frequency of litters (*1–3*). The families also show differences in the shape of the spleen (*4*), the length of the front legs (*5*), and in the distribution of cell types in the pituitary (*6*), and in certain physiologic reactions, such as the response to inoculation with tuberculosis bacilli (*7*, *8*), production of antibodies (*9*), and the survival of tissue transplants (*10*).

Relatively few spontaneous malignant neoplasms have been described in guinea pigs (*11*, *12*, *13*). Leader (*12*) in 1937 was able to assemble only 21 such tumors from the literature. No neoplasms have been observed in 15,000 guinea pigs of families 2, 13, and 35, or in other guinea pigs born and observed for as long as 5 years at Beltsville between 1916 and 1937.[2]

The early work on the experimental production of malignant neoplasms in guinea pigs was reviewed in 1931 by Maury (*11*), and more recently by Warren and Gates (*13*).

There are only four reports on the successful production of malignant tumors in guinea pigs following administrations of carcinogenic polycyclic aromatic hydrocarbons. Haagensen and Krehbiel (*14*) in 1936 induced liposarcomas and fibrosarcomas in 5 of 13 guinea pigs in 10 to 13 months after the subcutaneous injection of 3,4-benzpyrene dissolved in lard. Shear (*15*) mentions that subcutaneous tumors were obtained in two guinea pigs 14 and 20 months after the implantation of a cholesterol pellet containing 5 percent of 3,4-benzypyrene. Schabad (*16*) produced a fibrosarcoma and a liposarcoma in 2 of 7 animals, 19 and 22 months after the subcutaneous administration of 25

[1] *With the approval of Dr. John R. Mohler, Chief of the Bureau of Animal Industry.*

[2] *Eaton, O. N.: Personal communication (1940).*

to 48 mg. of 1,2,5,6-dibenzanthracene in sunflower-seed oil. Warren and Gates (*13*) obtained 2 fibrosarcomas and 2 liposarcomas in 32 guinea pigs which survived more than 11 months after a single subcutaneous injection of 5 mg. of 3,4-benzpyrene in 0.5 cc. of glycerin; the average induction time of these tumors was 16 months. Metastases, to the lungs, are described in only one instance, that of Schabad's liposarcoma (*16*).

The impression from the literature, therefore, is that although neoplasms can be induced in guinea pigs following injections of carcinogenic hydrocarbons, the low incidence and the long latent period of tumor induction classify the guinea pig among the animals which are relatively resistant to carcinogenesis with these agents.

EXPERIMENTAL PROCEDURE

Twenty-nine guinea pigs of families 2, 13, and 35, of both sexes and of various ages, were injected in the right axilla with 20-methylcholanthrene (m. p. 178.5°–179.2° C., corrected) dissolved in sesame oil, 10 mg. per cubic centimeter, on January 15, 1940. Half of the animals received a single dose of 20 mg., and half were injected in the same area four times at fortnightly intervals with 10 mg., or a total dose of 40 mg. of the compound. Six additional inbred guinea pigs were given a single subcutaneous injection of 20 mg. of methylcholanthrene on March 8, 1940.

On May 8, 1940, 10 stock guinea pigs of unknown genetic constitution were added to the series. These animals were injected once in the right axilla with 30 mg. of methylcholanthrene dissolved in 3 cc. of sesame oil.

The animals were maintained on a diet of rabbit pellets[3] daily, oats and hay three times a week, and cabbage and carrots three times a week; water was available at all times. They were weighed every 2 weeks for the first 6 months of the experiment, and were examined weekly for the presence of tumor at the site of injection.

Two inbred guinea pigs and two stock animals died of pneumonia, and one stock guinea pig died of fibrinous pericarditis within 4 weeks after the experimental procedure. These animals are not included in the results. Forty guinea pigs are considered here: 10 were of family 2, 13 of family 13, 10 of family 35, and 7 were stock animals. Individual data of the experiment are presented in tables 1 and 2.

Material for histologic examination was fixed in Zenker's fluid and in solution of formaldehyde (U. S. P., 1:10). Tissues that contained bone were decalcified in a solution of lactic and nitric acid. Some tissues were frozen and stained with sudan IV. The remainder were imbedded in paraffin, cut, and stained with haematoxylin and eosin, Mallory's phosphotungstic acid haematoxylin, van Gieson's stain, and Foot's silver stain for reticulum.

RESULTS

The action of methylcholanthrene, given subcutaneously in single 20-mg. doses or in four 10-mg. doses to guinea pigs, can be divided into (1) the general toxic effects and (2) the local carcinogenic effect.

General Toxic Effects

Reaction in family 2 guinea pigs

All four of the guinea pigs of family 2 which received the larger dose of methylcholanthrene died 11 to 15 weeks after the initial administration of the hydrocarbon. The syndrome was similar in all four: massive ascites, atrophy of the spleen, and hemorrhage in the liver. One guinea pig had hemorrhage in the adrenals and atrophy of the testes (table 1).

[3] *Maritime Mfg. Co., Buffalo, N. Y.*

TABLE 1.—*Effect of methylcholanthrene in guinea pigs of family 2*

Guinea pig No.	Sex	Age	Weight at injection	Methylcholanthrene injected	Death after injection	Hemorrhage in liver	Ascites	Atrophy of spleen	Hemorrhage in adrenal	Hemorrhage in kidney	Atrophy of testes
		Months	*Grams*	*Mg.*	*Weeks*						
1	M	6.6	692	[1] 40	15	+	+	+			
2	M	6.7	740	[1] 40	12	+	+	+			
3	M	3.0	539	[1] 40	11		+	+			
4	M	2.8	505	[1] 40	11	+	+	+	+		+
5	M	1.6	357	20	15	+		+			
6	M	1.6	355	20	11	+	+	+		+	+

[1] In 4 fortnightly injections of 10 mg.

TABLE 2.—*Individual data on guinea pigs*

Guinea pig No.	Family	Sex	Age at injection	Methylcholanthrene	Weight at injection	Weight 3 months after injection	Subcutaneous tumor first palpated, in weeks after injection	Killed or died, in weeks after injection	Weight of tumor at death	Histologic diagnosis of tumor [1]	Bone and/or cartilage in tumor	Pulmonary metastases	Transplantation
			Mos.	*Mg.*	*Grams*	*Grams*			*Grams*				
7	2	M	1.8	20	355	495		19					
8	2	M	2.0	20	450	698	22	38	100	Fibrosarcoma			+
9	2	F	2.0	20	340	529	28	38	55	do			+
10	2	F	3.7	20	481	588	18	30	?	do			
11	13	M	8.0	[2] 40	965	925	18	23	26	do			
12	13	M	8.1	40	1,000	874	24	28	7	do			
13	13	M	8.0	40	830	739	22	26	10	do			
14	13	M	8.1	40	970	932	18	31	65	do			
15	13	M	8.1	40	987	938	18	28	44	do			+
16	13	M	7.1	20	658	808	40	48	174	{1. Liposarcoma 2. Fibrosarcoma}			
17	13	M	6.1	20	1,068	1,102	24	34	225	Fibrosarcoma		+	
18	13	M	7.0	20	975	1,005	18	27	75	do			
19	13	M	7.2	20	745	795	24	36	120	{Liposarcoma Fibrosarcoma}	+	+	
20	13	M	7.5	20	948	933		[3]					
21	13	F	1.4	20	265	617	30	48	180	Liposarcoma			
22	13	F	3.6	20	375	562	27	33	47	{1. Liposarcoma 2. Fibrosarcoma}			+
23	13	F	1.4	20	280	538		[3]					
24	35	M	13.0	[2] 40	991	929	29	38	65	Fibrosarcoma	+		
25	35	M	5.7	40	700	675	18	28	12	do			+
26	35	F	13.0	40	827	774	22	28	65	do			+
27	35	F	13.0	40	725	684	20	28	22	{1. Liposarcoma 2. Fibrosarcoma}			
28	35	M	6.7	40	809	692	18	30	33	Fibrosarcoma			
29	35	M	1.9	20	391	727	32	38	22	{1. Liposarcoma 2. Fibrosarcoma}	+		
30	35	M	5.0	20	710	778	21	28	25	{1. Fibrosarcoma 2. do}			−
31	35	M	1.9	20	403	608	18	34	37	Fibrosarcoma			
32	35	M	4.1	20	670	754		37					
33	35	M	5.7	20	755	798	19	37	100	Fibrosarcoma		+	
34	Stock	F	5.6	30	873	905	28	30	10	{1. Fibrosarcoma 2. do}			
35	Stock	M	5-6	30	972	927	27	[4]		Fibrosarcoma	+		
36	Stock	M	5-6	30	887	919	27	32	45	{1. Fibrosarcoma 2. do}	+	+	
37	Stock	M	5-6	30	1,001	892	26	32	66	Liposarcoma			
38	Stock	F	5-6	30	754	878	29	36	40	{Liposarcoma Fibrosarcoma}			
39	Stock	F	5-6	30	644	771	28	32	100	Liposarcoma			
40	Stock	M	5-6	30	979	990		33					

[1] Numbered tumors represent multiple separate tumors; tumors in braces represent histologically mixed tumors.
[2] In fortnightly doses of 10 mg.
[3] Alive, without tumor, 52 weeks after injection.
[4] Alive, with tumor, 40 weeks after injection.

Two of six family 2 guinea pigs injected with 20 mg. of the carcinogen died in 11 to 15 weeks. Both had atrophy of the spleen and hemorrhage in the liver, and one had ascites, hemorrhage in one kidney, and testicular atrophy (table 1).

A description of the clinical course and the pathologic findings of guinea pig No. 1 is characteristic of the reaction of the family 2 guinea pigs to methylcholanthrene. About 11 weeks after the first injection of methylcholanthrene the guinea pig appeared ill and lost weight. During the following 2 weeks it became emaciated and the abdomen became distended. Clear straw-colored fluid which contained rare lymphocytes and 1 percent of protein was obtained by abdominal paracentesis. The animal died 2 weeks later.

At necropsy, the abdominal cavity contained over 200 cc. of clear, reddish fluid. The liver was not enlarged. It was firm, granular, brown-red, with depressed gray or black subcapsular areas, varying from a few millimeters to 1 cm. in diameter. These areas were soft and extended into the substance of the liver. The spleen was small, measuring $3 \times 1.2 \times 0.6$ cm., red, rubbery, and the markings were obscured.

Histologic sections of the liver revealed that the hepatic cells near the portal areas were coarsely granular and hypertrophic, while those around the central areas were vacuolated. The portal spaces were larger than usual and the number of bile ducts and the amount of portal connective tissue were increased. Focal extravasations of blood obliterated entire lobules in some areas. No evidences of thrombosis or inflammatory reaction were seen (pl. 1, *A*).

The spleen was reduced to a network of large vascular channels among which few small follicles were seen. Phagocytosed pigment was abundant and the trabeculae were prominent (pl. 1, *B*).

This syndrome, characterized chiefly by ascites, intrahepatic hemorrhage, and splenic atrophy, was not seen in any guinea pigs of families 13 and 35, or in the stock animals. Neither was the syndrome observed in four guinea pigs of family 2 which survived the injection of 20 mg. of methylcholanthrene. Fatty change in the liver was encountered frequently in guinea pigs of all families, but the acute reaction was limited to the animals of family 2.

This syndrome was apparently the same as described by Schabad and Urinson (*17*) in 15 of 18 stock guinea pigs within 2 to 11 months after the subcutaneous or intraperitoneal injection of 25 to 48 mg. of 1, 2, 5, 6-dibenzanthracene dissolved in sunflower-seed oil. Haagensen and Krehbiel (*14*) did not mention a similar effect following the administration of 3, 4-benzpyrene. That the reaction does not develop with the latter compound is also suggested by Polson's work (*16*) with rabbits, in which 1, 2, 5, 6-dibenzanthracene produced hepatic necrosis whereas benzpyrene did not.

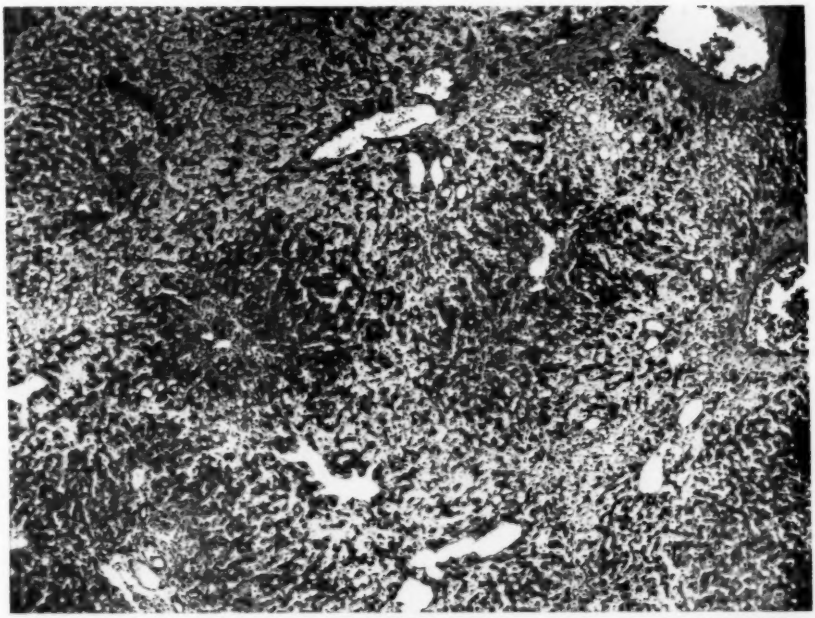
Effect on weight

The deleterious effect of methylcholanthrene given in four doses of 10 mg. each was shown by observations on the weights of guinea pigs of families 13 and 35. The effect was obscured in the animals of family 2 by the presence of ascites, and this group was not considered. Every guinea pig of families 13 and 35 which received this dose of the hydrocarbon lost weight during the ensuing 3 or 4 months. Otherwise the general condition appeared good. After 4 months the animals apparently began to regain weight, although this was obscured by the appearance of tumors.

Guinea pigs of families 13 and 35 which received 20 mg. of the hydrocarbon in one injection continued to gain weight normally, as compared with the weights on normal animals of these families compiled by McPhee and Eaton (*3*).

PLATE 1

A



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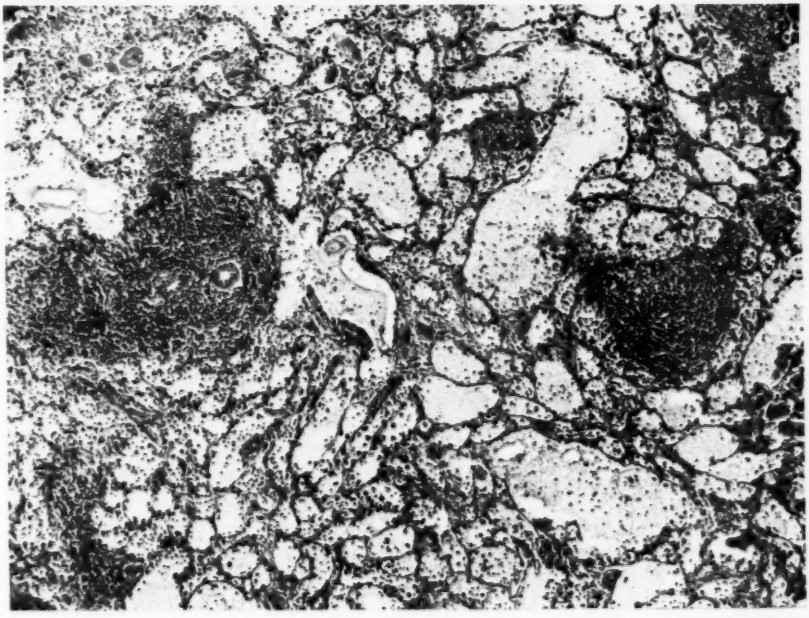

A, Liver of family 2, guinea pig No. 1, 15 weeks after subcutaneous injection of methylcholanthrene, showing extravasated blood, distended blood vessels, and focal areas of fatty infiltration. Haematoxylin and eosin, ×60; *B*, Spleen from same animal. The sinusoids are distended and the follicles are atrophic. Haematoxylin and eosin, ×60.

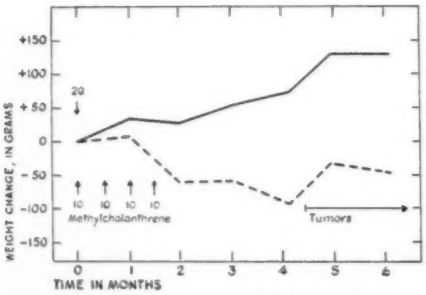

FIGURE 1.—Effect of methylcholanthrene on weight of guinea pigs. Solid line: 20 mg. in one injection. Average of eight animals, Nos. 16–20, 30, 32, 33 (table 2). Broken line: 40 mg. in four injections of 10 mg. each. Average of nine animals, Nos. 11–15, 24–27 (table 2).

Figure 1 summarizes the effect of methylcholanthrene on the body weight of guinea pigs. Only adult guinea pigs weighing 700 to 1,000 gm. were included.

This effect on weight may have been due in some measure to the cumulative action of the compound given in four injections. Young guinea pigs of families 13 and 35, weighing 265 to 400 gm., continued to gain weight normally after a single injection of 20 mg., or an equivalent of 40 mg. given to the older animals if the total dose and the dose per body weight are considered.

There was no definite effect on weight in the stock animals injected with 30 mg. of methylcholanthrene. In 3 months, three of the seven guinea pigs had lost weight and four had gained.

No determinations of the food intake were made. Whether the effect on weight was due to loss of appetite or to some other action of the injected chemical, therefore, is not known.

LOCAL CARCINOGENESIS

About 16 weeks after the first injection of methylcholanthrene, 10 of 34 guinea pigs which survived had thickening of the subcutaneous tissue at the site of injection. Two weeks later, 7 of these animals had definite hard masses which were circumscribed and fairly movable and which continued to increase in size.

During the following 7 months, 29 of 34 guinea pigs developed tumors at the site of injections, as summarized in tables 2 and 3. In 7 instances two separate and discrete masses arose at the same time. The multiple tumors were not attributable to multiple injections of methylcholanthrene, since only one of the seven arose in animals which received more than one injection. The relatively large amount of solvent (2 to 4 cc.) probably permitted the contact of the carcinogen with a wide area of tissue.

Biopsy of three masses was performed 22 weeks after injection. The tumors were found to be fibrosarcomas (Nos. 11, 18, and 30, table 2).

The growth rate of the tumors varied widely. One tumor (No. 25) attained a

TABLE 3.—*Induction of tumors in guinea pigs injected subcutaneously with methylcholanthrene in sesame oil*

Family	Methylcholanthrene	Animals	Tumors, in weeks after injection									Total tumors	Average latent time	Type of tumor [1]		
			18	20	22	24	26	28	30	32	36+			Fibrosarcoma	Liposarcoma	Bone or cartilage formation
	Mg.	*Number*										*Number*	*Weeks*			
2	20	4	1		1			1				3	22.7	3		
13	[2] 40	5	3		1	1						5	20.0	5		
13	20	8	1			2		1	1		1	6	27.2	5	4	1
35	[3] 40	5	2	1	1				1			5	21.4	5	1	1
35	20	5	1	1	1					1		4	27.0	5	1	1
Stock	30	7					1	4	1			6	27.5	6	3	2

[1] 9 guinea pigs had more than 1 tumor.

[2] Injected in 4 fortnightly doses of 10 mg.

weight of 12 gm. during 10 weeks of observation, whereas another histologically similar tumor (No. 17) reached 225 gm. during the same period. Once a tumor was palpable it grew progressively. No regression occurred, although several tumors became softer due to central necrosis. In the larger fibrosarcomas there was considerable central necrosis, and upon section liquefied tissue and serosanguinous fluid often exuded. In four animals with large tumors there was ulceration and bleeding.

No evident differences in the susceptibility to local carcinogenesis with methylcholanthrene were demonstrable in the guinea pigs of the inbred families, and the stock heterozygous guinea pigs were as susceptible as the inbred animals (table 3). Although the high doses of the carcinogen may have obscured the influence of the strain differences, the close agreement in the latent periods of the palpable tumors in the groups at the dose levels used, permits the tentative conclusion that the three strains are approximately equal in susceptibility to subcutaneous carcinogenesis with this agent.

Comparable male and female guinea pigs developed tumors at the same average latent time of 25.5 weeks. Six young guinea pigs, up to 2 months of age, developed tumors 24.8 weeks after injection, whereas six older animals, about 6 months old, which received the same dose of methylcholanthrene, had tumors in an average of 24.3 weeks.

The combined incidence and latent period data of this series can be used in comparing the susceptibility of the subcutis of the guinea pig to other carcinogenic hydrocarbons, as reported in the literature (*13, 14, 16*). Table 4 shows that the relative carcinogenic potency of 20-methylcholanthrene, 3, 4-benzpyrene and 1, 2, 5, 6-dibenzanthracene in guinea pigs is the same as in mice and in rats (*19*), but that guinea pigs are more resistant to the induction of subcutaneous neoplasms with these agents.

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Description of Tumors

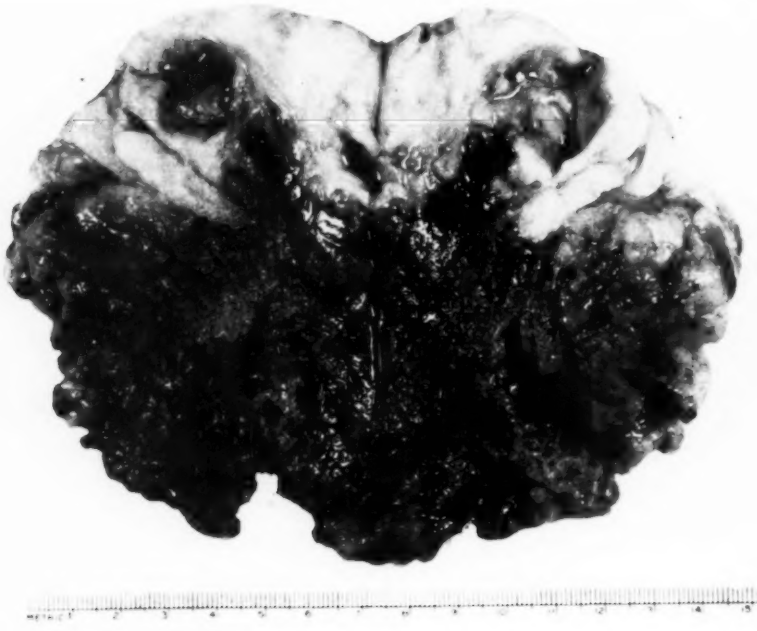
The tumors that arose at the site of injection of methylcholanthrene were of two general types: fibrosarcoma or liposarcoma. Of the 38 tumors that appeared, considering 9 instances of mixed or of multiple tumors as separate neoplasms, 29 were fibrosarcomas and 9 were liposarcomas. Of the liposarcomas, 4 occurred in guinea pigs of family 13, and 2 in animals of family 35. There was some indication that liposarcomas appeared later than the fibrosarcomas, since the average latent period for 9 liposarcomas was 28.4 weeks, whereas a comparable group of 8 fibrosarcomas became palpable 22.7 weeks after injection.

Fibrosarcomas

The fibrosarcomas were gray-white, spheroidal, circumscribed, subcutaneous masses. They were usually fixed to the overlying skin but could be enucleated from the deeper tissues which frequently appeared edematous. Small tumors were hard. The cut surface was gray and fibrous. Larger fibrosarcomas ulcerated through the skin, were elastic in consistency, and contained a necrotic core surrounded by a rim of firm, gray, fibrous tissue (pl. 2).

Table 4.—*Response of guinea pigs to subcutaneously injected carcinogenic hydrocarbons*

Hydrocarbon	Dose	Solvent	Guinea pigs	Tumors	Average latent time
	Mg.		*No.*	*No.*	*Mos.*
20 - Methylcholanthrene.	40	Sesame oil	10	10	5.0
Do	30	do	7	6	6.5
Do	20	do	17	13	6.5
3,4-Benzpyrene (*14*)	35	Lard	13	5	11.5
Do. (*13*)	5	Glycerin	32	4	16.0
1,2,5,6 - Dibenzanthracene (*16*).	25–48	Sunflowerseed oil.	7	2	20.5



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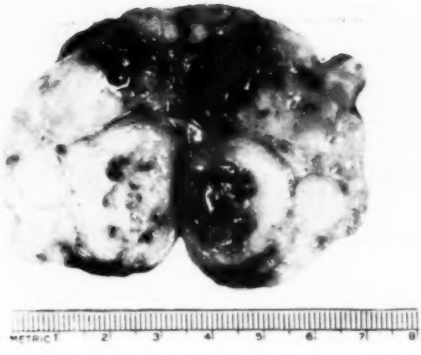


C

A, Cut surface of large axillary fibrosarcoma with extensive necrosis. Guinea pig No. 17, family 13; *B*, Lung of guinea pig No. 17, showing pulmonary metastases ×3; *C* Cut surface of axillary fibrosarcoma. Guinea pig No. 31, family 35.

The cell population varied widely within each neoplasm. The majority of the cells resembled atypical fibroblasts with fibrillar processes and elongated nuclei. They varied in size and were frequently arranged in compact interlacing bundles or broad sheets. Fibroglia, appearing as fine, intracellular purple fibrils, were seen in many of the neoplastic cells when stained with Mallory's phosphotungstic acid haematoxylin. Collagen, as demonstrated by van Gieson's and Mallory's phosphotungstic acid haematoxylin stains, was moderately abundant in these areas. Mitotic figures and tumor giant cells were seen. Some regions contained short, broad, spindle-shaped cells mixed with round or irregular forms. These had a moderate amount of acidophilic cytoplasm, hyperchromatic nuclei, no processes, and showed no characteristic structural arrangement. Collagen was rarely demonstrated among them, but mitotic figures and tumor giant cells were numerous. Hyalinized collagen, inflammatory reaction, hemorrhage, edema, and necrosis were seen in some tumors. The blood vessels were usually represented by channels in the neoplastic tissue lined by a single layer of endothelium (pl. 3.)

The fibrosarcomas infiltrated contiguous tissues. Three of them metastasized to the lungs where they reproduced the structure seen in the more undifferentiated parts of the parent tumor.

Liposarcomas

Most of the liposarcomas were soft, yellow, encapsulated, subcutaneous masses. Large ones were fluctuant. On incising the capsule semisolid material which could not be grasped with forceps exuded from some. Others were firmer and contained some fibrous tissue. Macroscopic necrosis was rarely seen.

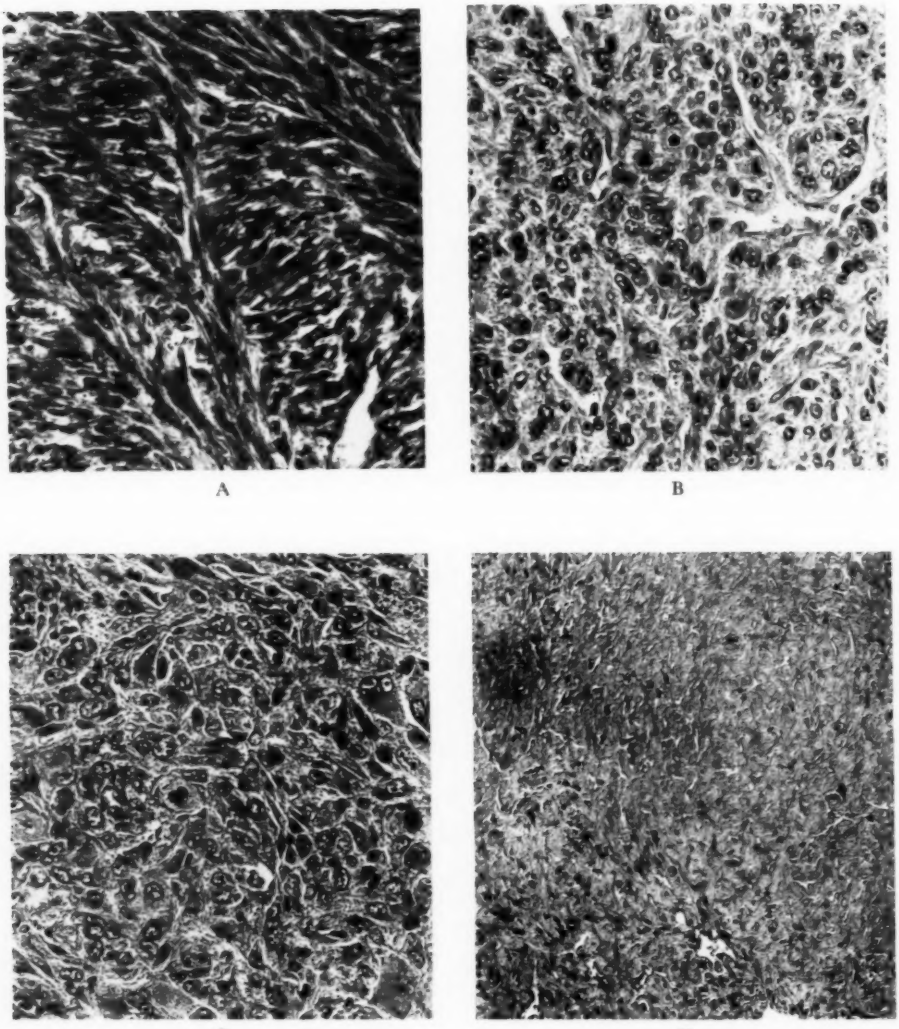
Some variation in histologic structure existed among the liposarcomas. The cells formed broad sheets in the peripheral parts of the tumors, but the central portions appeared loosely constructed and edematous. Small foci of necrosis were seen occasionally. The cells were generally ovoid or polygonal and varied in size. The pale acidophilic cytoplasm usually contained one or more vacuoles which stained red with sudan IV. The nuclei were granular. Some cells consisted of a large fat vacuole surrounded by a thin rim of cytoplasm containing a flattened nucleus. They resembled "signet ring" cells when stained with haematoxylin and eosin. One neoplasm (No. 16, pl. 4, *D*) had a large area of closely packed, small, ovoid cells with granular nuclei. Many of these cells contained minute, red, cytoplasmic vacuoles when stained with sudan IV, although no vacuolation was detected in preparations stained with haematoxylin and eosin. Other sections from the same tumor were composed of sheets of coarsely vacuolated cells.

Mitotic figures and tumor giant cells were present in all of the liposarcomas. The capsules were formed by dense fibrous tissue (pl. 4).

One liposarcoma metastasized to the lungs. The metastases appeared as gray, circumscribed, subpleural nodules which, on histologic examination, were made up of closely packed polygonal cells with round nuclei and finely vacuolated cytoplasm.

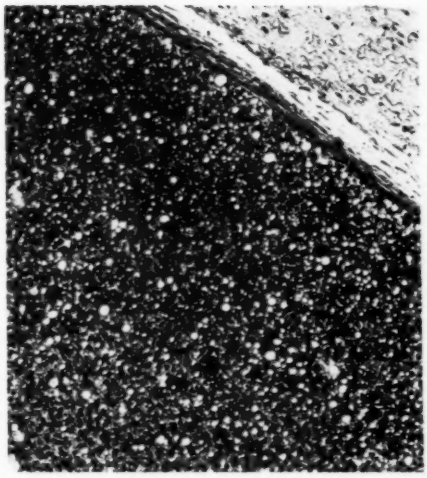
The frequent occurrence of liposarcomas in guinea pigs injected with carcinogenic hydrocarbons was recorded by previous investigators. Of 14 tumors in 11 guinea pigs studied by Haagensen and Krehbiel (*14*), Schabad (*16*), and Warren and Gates (*13*) 7 were liposarcomas. As Haagensen and Krehbiel stated, subcutaneous injection in guinea pigs must deposit the hydrocarbon in a thick panniculus of fat which the guinea pig possesses.

PLATE 3



A, Fibrosarcoma from guinea pig No. 8, family 2. The cells resemble atypical fibroblasts and form interlacing bundles. Haematoxylin and eosin, ×260; *B*, Another area from the same tumor, showing less differentiation than in (A). Haematoxylin and eosin, ×260; *C*, Pleomorphic area from tumor in guinea pig No. 19, family 13. Cells vary greatly in size, shape, and staining characteristics. Haematoxylin and eosin, ×260; *D*, Area of hyalinized collagen in fibrosarcoma of guinea pig No. 17, family 13. Haematoxylin and eosin, ×260.

PLATE 4



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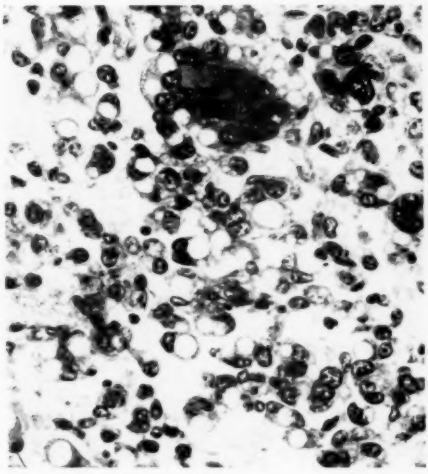

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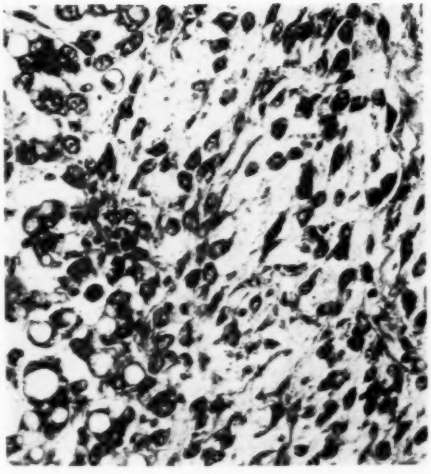

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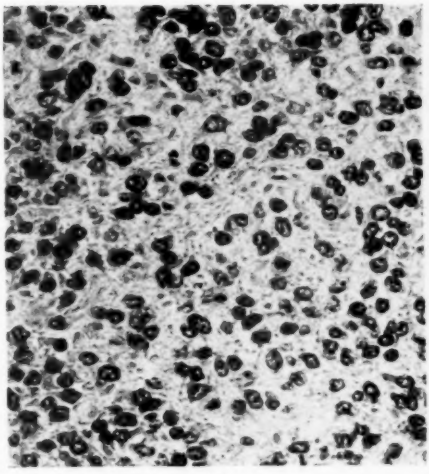


D

A, Liposarcoma in guinea pig No. 22, family 13, showing highly cellular character of growth and well-defined capsule. Haematoxylin and eosin, ×80; *B*, Higher magnification of same tumor, showing "signet-ring" and giant cells. Haematoxylin and eosin, ×260; *C*, Mixed fibrosarcoma and liposarcoma, in guinea pig No. 19, family 13. Haematoxylin and eosin, ×260; *D*, Area of undifferentiated tissue in liposarcoma in guinea pig No. 16, family 13. Haematoxylin and cosin, ×260.

Neoplasms containing bone or cartilage

Five guinea pigs developed bone or cartilage in the tumors. The average latent period of these tumors was 27.8 weeks.

Guinea pig No. 19: A fusiform mass, 8.5 cm. long and 3.5 cm. in greatest diameter, weighing 120 gm., grew in the foreleg and eroded a small area of the shaft of the humerus. It was firm and elastic in consistency and had a yellow-gray, glistening, cut surface. An area adjacent to the erosion in the humerus appeared lighter and more fibrous than the surrounding tissue. It contained an irregular, chalky white, calcified nodule which looked like an area of calcified fat necrosis.

Areas of liposarcoma, fibrosarcoma, and a mixture of both were seen on histologic examination. Some regions of the neoplasm appeared edematous. They contained small foci of atypical fat cells surrounded by spindle-shaped or stellate cells with long fibrillar processes that stained red with van Gieson's stain and Mallory's phosphotungstic acid haematoxylin.

The calcified nodule contained cartilage and bone. It was surrounded by fibrosarcoma. The cartilage cells were ovoid, had abundant acidophilic cytoplasm, eccentrically placed nuclei, and lay in lacunar spaces within a hyaline matrix. The cells were most numerous near the edges of the chondroid masses. In some areas the cartilage lay next to bone without any intervening fibrous tissue. No evidence of endochondral ossification was found.

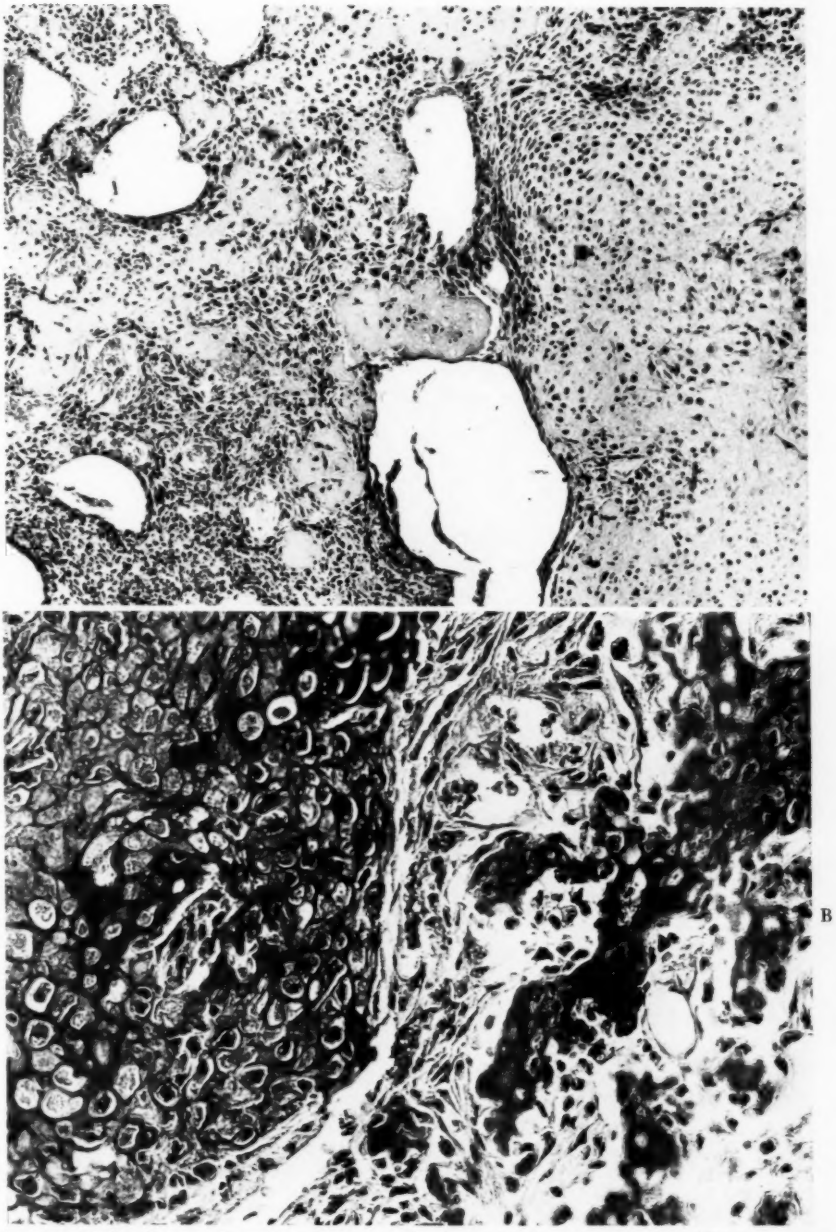
Cellular tissue formed a thick zone at the edge of the cartilage. It contained atypical chondroblasts, spindle cells, and tumor giant cells. A tendency to whorl formation was noted. Mitotic figures were numerous in this area but were seen rarely in other parts of the calcified nodule.

The bone consisted of closely packed spicules with little interosseus tissue. The larger spicules contained bone cells. Many were surrounded by a single layer of hyperchromatic pyramidal or flattened cells that looked like osteoblasts. A few multinucleated cells resembling osteoclasts were also seen. Large, thin-walled blood vessels were present among the bony spicules. An area of necrosis was seen in the center of the osseous tissue. It was not associated with an inflammatory reaction. Another histologic section taken near the eroded area of the humerus showed calcified osteoid tissue. Peripheral parts of this tissue contained edematous sheets of spindle-shaped cells. The central portion of the section contained irregular deposits of calcium surrounded by round or polygonal cells imbedded in a dense, acidophilic fibro-hyaline matrix. The calcified foci contained no bone cells, osteoblasts, or osteoclasts. A zone of pleomorphic cells varying greatly in size lay between the peripheral and central portions of the sections. Bizarre nuclei were seen in many of these cells. Mitotic figures were not numerous. Very few blood vessels were present (pl. 5).

This neoplasm metastasized to the lungs. The metastases were circumscribed masses of polygonal cells with finely vacuolated cytoplasm. No evidence of cartilage or bone was found in the pulmonary metastases.

Guinea Pig No. 36: This animal had two axillary masses. The larger of these weighed 40 gm. and measured 5.6×3.8 ×4.4 cm. It was coarsely granular, circumscribed, and hard. The white fibrous cut surface was divided into lobules by dense trabeculae. This neoplasm, a fibrosarcoma, was composed of long spindle-shaped cells with fibrillar processes. They were arranged in interlacing bundles, loosely constructed edematous sheets, and in compact masses associated with dense,

PLATE 5



A, Area containing bone and cartilage in tumor of guinea pig No. 19, family 13. Haematoxylin and eosin, ×80; *B*, Higher magnification of the same tumor, showing spicules of bone and cartilage cells. Mallory's phosphotungstic acid haematoxylin, ×260.

collagenous, intercellular substance. Small foci of more undifferentiated cells were also seen. Mitotic figures and tumor giant cells were present. The neoplastic tissue invaded skeletal muscle.

The smaller mass lay between the fibrosarcoma and the thoracic wall. It was enucleated readily from the surrounding tissue. The tumor was smoothly outlined, gray, and stony hard. The cut surface was pitted and granular. This neoplasm contained numerous masses of imperfectly formed cartilage imbedded in fibrosarcoma. The cartilage cells varied greatly in size and shape. Some of them resembled chondrocytes; some had large, oval, granular nuclei with scanty cytoplasm; some were triangular in shape; and some were multinucleated. Most of the cells lay in well-defined lacunae, but the character of the matrix varied from homogenous hyaline substance to scanty granular or fibrillar material. Lacunar spaces containing two cells were seen frequently. Mitotic figures were rarely found among the cartilage cells. The perichondrial tissue contained closely packed, short, spindle-shaped, and large, ovoid cells among which numerous mitotic figures were seen. Giant cells of the foreign body type were found in a few areas. In one field the cartilage appeared to be partially calcified and a few small bony spicules were seen (pl. 6).

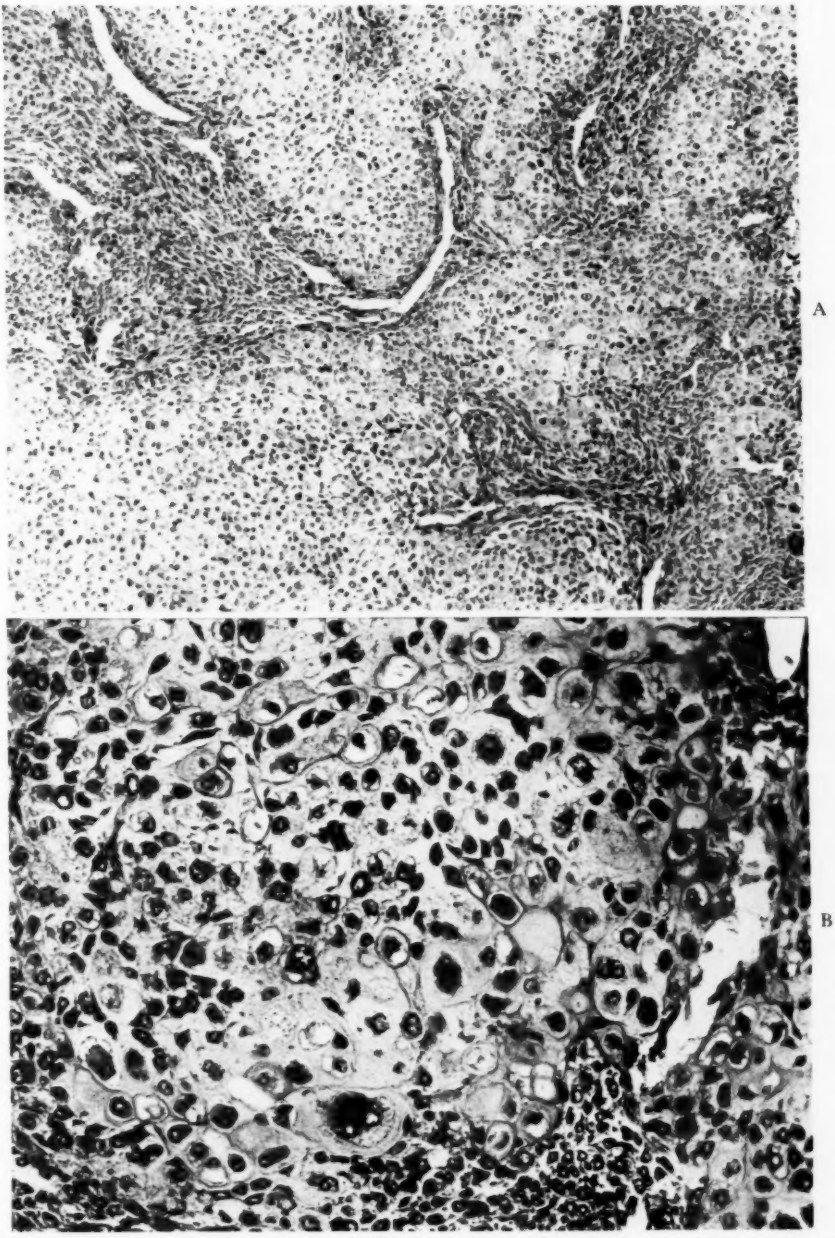
A section of the lung from this guinea pig contained small circumscribed, compact, intravascular masses of moderately large ovoid or polygonal cells with well-defined cell membranes. These probably represented tumor metastases.

Guinea pig No. 24: A circumscribed firm axillary mass weighed 65 gm. and measured 4.4×4.4×5.5 cm. The cut surface was white and fibrous. The central portion was necrotic. A calcified nodule was seen in the periphery of the tumor beneath the subcutaneous tissue at least 4 cm. from the nearest bone.

Much of the neoplasm was composed of loosely constructed sheets and interlacing bundles of atypical spindle-shaped cells with long fibrillar processes. Hyalinized collagen and necrosis were present. Mitotic figures and tumor giant cells were seen. The calcified nodule was made up of small, dense, acidophilic spicules. Some of them were surrounded by deeply staining, small, pyramidal, or cuboidal cells resembling osteoblasts. No bone cells or osteoclasts were seen. The area appeared to be more vascular than the rest of the neoplasm. Some of the blood vessels had thick, edematous walls. Two small foci of necrosis were present in the nodule, but they were not associated with inflammatory reaction. The neoplastic tissue adjacent to the area of bony spicules was osteoid. The cells were small, pleomorphic, and had pale cytoplasm with irregular, granular nuclei. They lay in a thin fibrillar network. Occasional spicules of bone were seen. Few mitotic figures and no giant cells were found in these regions. No cartilage was seen in any sections of the tumor that were studied (fig. 2).

Guinea pig No. 29: The fourth neoplasm associated with bone formation was a small, soft, subcutaneous, yellow liposarcoma of the axilla. No macroscopic calcification was present. A minute area containing regular spicules of well-formed bone imbedded in fibrosarcoma was seen on histologic examination. The osseous focus lay at the periphery of the liposarcoma and was separated from it by a fibrous capsule. No cartilage was present. The bony spicules were surrounded by cells that resembled osteoblasts. The interosseous tissue was composed of stellate and spindle-shaped cells. No mitotic figures or tumor giant cells were encountered.

PLATE 6



A, Area of atypical cartilage and spindle cells in stock guinea pig tumor No. 36. Haematoxylin and eosin, ×80; *B*, Area of atypical hyaline cartilage from the same tumor, showing marked variation in cell size. Haematoxylin and eosin, ×260.

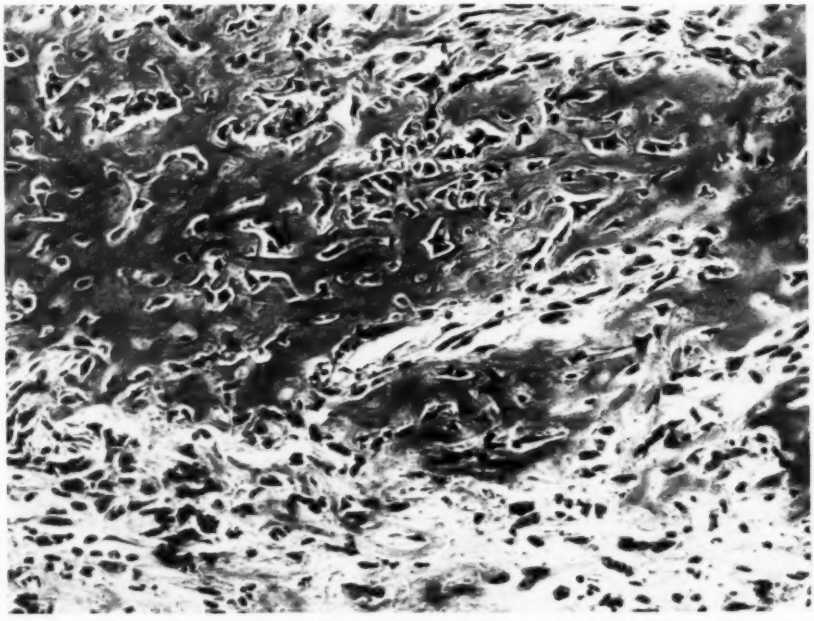


FIGURE 2.—Area of well-formed bone in fibrosarcoma. Guinea pig No. 24, family 35. Mallory's phosphotungstic acid haematoxylin, ×260.

Guinea pig No. 35: The fifth guinea pig which developed a calcified mass is still alive. Biopsy at 37 weeks after injection showed the mass to be partly a fibrosarcoma. Serial roentgenograms demonstrated an extraskeletal area of increased density consistent with calcification. The area is growing progressively.

The nature of the bone, cartilage, and osteoid tissue in these neoplasms is debatable. Many of the morphologic characteristics of the calcified area of the tumor from guinea pig No. 19 suggest true osteogenic sarcoma. Support for this contention is afforded by the erosion of the humerus adjacent to the osteogenic tissue. None of the other neoplasms invaded bone although many of them lay close to the pectoral girdle or the ribs. The cartilage seen in the tumor of guinea pig No. 36 also appeared neoplastic, yet the chondral masses were completely surrounded by fibrosarcoma and showed no invasive qualities. The bone spicules found in the two remaining neoplasms appeared well formed and regular. They resembled the spicules seen in callus or in membranous bone formation rather than the tumor bone of osteogenic sarcoma.

Mesenchymal neoplasms containing bone and cartilage have been described in man, dogs, rats, and mice. Heterotropic bone may be formed by metamorphosis of adult collagenous tissue. Recent work on the subject by Binkley and Stewart (*20*) and by Allen (*21*) stresses the importance of dense hyalinized collagen as a matrix for calcium inpregnation. Circulatory stasis, trauma, stress, strain, necrosis, and inflammatory reaction have been thought to play some role in the transformation of collagen to bone. The bone in the guinea-pig tumors was surrounded by fibrosarcoma in which rela-

tively adult, collagen-forming cells predominated. An osteoid tissue containing collagen fibrils or collagenous matrix sometimes intervened between the atypical, neoplastic fibroblasts and the bone spicules. In general the bone forming areas suggested "parts left behind in the course of active growth" (*20*) in contrast with the proliferating fibrosarcoma. Homogenized collagen and edema were seen frequently in fibrosarcomas whether or not they contained bone. The osteogenic areas appeared less vascular than the surrounding tissue in most cases. The tumor from guinea pig No. 19, however, contained large, thin-walled blood vessels among the bone spicules. Neither hemorrhage nor inflammatory reaction were seen in any of the bone-forming regions or contiguous areas. Simple necrosis of the center of the calcific nodule was present in two neoplasms. No evidence of necrosis was found in the peripheral part of the bone-forming area or in the adjacent tumor.

Whether cartilage may be formed by intramembranous chondrification is disputed. Allen (*21*) favored this mechanism. Binkley and Stewart (*20*) believed that the morphologic changes observed in ossifying neoplasms did not explain the structure of cartilage-containing mixed tumors. Our observations do not clarify the problem. The highly cellular areas interposed between cartilage and fibrosarcoma in the tumor from guinea pig No. 19 suggested a transitional zone of chondroblastic cells. The neoplasm from guinea pig No. 36 contained large amounts of cartilage that lay next to atypical fibroblasts without any evidence of a transitional zone.

Bone and cartilage formation in guinea-pig tumors may afford an opportunity for investigating the mechanism of heterotropic bone and cartilage formation in neoplasms. Further studies of this phenomenon are now in progress.

Metastases

Metastases, confined to the lungs, were observed in four guinea pigs. In three instances the metastases were derived from a fibrosarcoma, and in one the growths were secondary to a liposarcoma.

Many of the animals were killed early, and family 35 guinea pigs, which are particularly susceptible to pneumonia, often died while the tumor was of relatively small size. It is probable that the incidence of metastases would have been higher had all the animals been kept until natural death. No metastases were encountered in 10 guinea pigs whose tumors weighed less than 40 gm.; however, in 6 animals the tumor weighed over 100 gm., and no metastases were present. Although the average survival after the appearance of a palpable tumor in 4 animals with metastases was 11 weeks, 1 had pulmonary metastases only 5 weeks after the detection of a tumor at the site of injection (No. 36). The average survival of guinea pigs which did not have metastases was about 8 weeks, but 5 lived for over 12 weeks.

Transplantation

Seven tumors that arose in inbred guinea pigs were transplanted into the subcutaneous tissue of animals of the same family as the host of the original tumor.

Four fibrosarcomas were transplanted by the trocar technique, in which a small solid piece of tumor tissue was used. Of these, two grew successfully (Nos. 15 and 26), although the latter began to grow 14 weeks after transplantation. One tumor (No. 25) appeared 17 weeks after inoculation in one guinea pig out of the three into which it was introduced. The fourth tumor (No. 30) failed to grow in three animals of family 35.

Three tumors, two fibrosarcomas and one liposarcoma, were transplanted by the mash technique, in which about 0.3 cc. of mashed tumor was injected through a needle. Of these, two (No. 9 and the liposarcoma of No. 22) grew within 4 weeks. The third tumor (No. 8) grew in one out of three guinea pigs of family 2 into which it was injected.

Four of the above tumors which grew in animals of the same family as the host of the original tumor were transplanted also into guinea pigs of other families. No growth was observed in 26 weeks. The liposarcoma (No. 22) was transplanted in the second passage into nine stock guinea pigs and grew in two of them.

SUMMARY

Forty guinea pigs, of inbred families 2, 13, and 35, and stock guinea pigs, were injected subcutaneously with 20 to 40 mg. of 20-methylcholanthrene dissolved in sesame oil.

Thirty-four animals survived over 18 weeks, and 29 developed tumors at the site of injection. The tumors became palpable in 18 to 40 weeks, or in an average of 25 weeks after injection. Seven guinea pigs developed 2 separate subcutaneous tumors, and in 2 the tumors were of a mixed type.

Of the 38 tumors in 29 guinea pigs, 29 were fibrosarcomas, and 9 were liposarcomas; 5 tumors contained bone or cartilage. Four tumors metastasized to the lungs, and 6 of 7 tumors were transplanted into guinea pigs of the same family as that in which the tumor arose.

There were no strain differences in susceptibility, under the conditions of the experiment, except that family 2 guinea pigs developed ascites and hepatic and splenic lesions following injection of 40 mg. of methylcholanthrene; in families 13 and 35 there was significant weight loss with this dose of the hydrocarbon.

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